# Multimodal Regulation of the Oxygen Sensing Pathway by Presenilin Membrane Proteases

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**von**


## Muriel Rahel Kaufmann

**von Sursee LU**


**Promotionskomitee**

Prof. Dr. Roland H. Wenger (Vorsitz)
PD Dr. Gieri Camenisch (Leitung der Dissertation)
Prof. Dr. Roger Nitsch
Dr. Ester M. Hammond

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This work has been performed under the supervision of

PD Dr. Gieri Camenisch and
Prof. Dr. Roland H. Wenger

at the Institute of Physiology and Zürich Center for Integrative Human Physiology (ZIHP), University of Zürich, CH-8057 Zürich, Switzerland

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## Summary

This dissertation is divided into two parts. The first project addresses the role of presenilin 1 and 2 in the regulation of the oxygen sensing pathway. In the second part, we investigated a potential role for the onconeural cerebellar degeneration related antigen Cdr2 as a novel tumor marker in papillary renal cell carcinoma. The two projects will be described and discussed separately, with a general introduction about hypoxia at the beginning.

The availability of free oxygen on Earth was one of the prerequisites for the evolution of eukaryotic life. Multicellular organisms need oxygen to efficiently produce ATP. Therefore, mechanisms for adequate adaptation to low oxygen environments (hypoxia) was developing during evolution. The main research interest of our group is to understand the molecular and cellular mechanism of sensing and adaptation to hypoxic environments.

Hypoxia is a driving force in many physiological and pathophysiological processes such as high mountain climbing, red blood cell synthesis, wound healing, cancer progression and neurodegenerative disorders.

The molecular sensors of hypoxia are the prolyl-4-hydroxylase domain containing proteins (PHDs) that regulate the degradation of the hypoxia-inducible factors $\alpha$ (HIFs$\alpha$) via oxygen dependent hydroxylation. In normoxia, the von Hippel-Lindau tumor suppressor protein (pVHL) binds to hydroxylated HIF$\alpha$ which leads to polyubiquitination and subsequent proteasomal destruction. In hypoxia, the cofactor oxygen is limited and the hydroxylation reaction is therefore reduced. The stabilized HIF$\alpha$ subunit then translocates to the nucleus and forms a potent transcription factor together with its constitutive counterpart HIF$\beta$. HIF regulates the expression of more than 200 genes that are involved in the adaptation to low oxygen partial pressure. Apart from oxygen-dependent HIF$\alpha$ protein regulation, there is growing evidence that HIF$\alpha$ mRNA is regulated by oxygen-independent mechanisms, for example by pro-inflammatory stimuli such as nitrogenmonoxide or cytokines.

*Project I:*

Recently, we have shown that the peptidyl/prolyl cis/trans isomerase FKBP38 specifically interacts with PHD2 and negatively regulates its protein stability (Barth *et al.* 2007). A report suggesting that presenilin 1 and 2 (PSEN1/2) interact with FKBP38 and are involved in the negative regulation of FKBP38 protein levels (Wang *et al.* 2005) draw our attention to a potential role of PSEN1/2 in PHD/HIF signaling.

Presenilin (PSEN) 1 and 2 are the catalytic components of the γ-secretase complex which cleaves a variety of proteins, including the amyloid precursor protein (APP). Proteolysis of APP leads to the formation of the APP intracellular domain (AICD) and amyloid beta (Aβ) that is crucially involved in the pathogenesis of Alzheimer's disease.

We found that PSEN1/2-deficient mouse embryonic fibroblasts (MEFs) express more FKBP38 protein that leads to reduced constitutive PHD2 protein levels. While decreased PHD2 levels would predict an increased hypoxic response, we interestingly found the opposite. Increased FKBP38 protein levels and decreased PHD2 protein levels were found in PSEN1/2-deficient mouse embryonic fibroblasts (MEFs) and in the cortex of forebrain-specific PSEN1/2 conditional double knock-out mice and resulted in a blunted hypoxic induction of specific HIF-target genes. While HIF-1α protein stability was not altered, we found decreased HIF-1α mRNA levels as well as reduced HIF-1α promoter activity in the absence of PSEN1/2. Proteolytic γ-secretase function of PSEN1/2 was needed for proper HIF activation. Intriguingly, PSEN1/2 mutations identified in Alzheimer patients differentially affected the hypoxic response, involving the generation of AICD. Taken together, our results suggest a direct role for PSEN in the regulation of the oxygen sensing pathway via the APP/AICD cleavage cascade.

Little is known about the biological function of PSEN1/2 and our data might help to elucidate the physiological role of PSEN1/2 and the γ-secretase complex. Additionally, the number of Alzheimer's patients is steadily growing with increasing life expectancy. Hypoxic regions in the brain correlate with pathologic protein deposits but the mechanistic connection is still unclear. Therefore, our work may contribute to the understanding of the role of hypoxia in the pathology of neurodegenerative diseases.

*Project II:*

Neoplastic expression of the onconeuronal cerebellar degeneration-related antigen Cdr2 in ovary and breast tumors is associated with cerebellar degeneration. Cdr2 protein expression is normally restricted to neurons, but tumoral Cdr2 expression triggers tumor immunity, thereby constraining tumor growth but also resulting in paraneoplastic cerebellar degeneration (PCD). So far, aberrant Cdr2 expression has mainly been described for breast and ovarian tumors. We found strong Cdr2 protein expression in 54.2% of papillary renal cell carcinoma (pRCC) and to a much lesser extent (7.8%) in clear cell (cc) RCC (Balamurugan *et al.* 2009). We identified Cdr2 as a novel PHD1 interacting protein and found increased PHD1 protein levels when Cdr2 was concomitantly overexpressed. High Cdr2 protein levels correlated with decreased HIF target gene expression in cells as well as in pRCC, providing a possible explanation why pRCCs are the most hypovascular renal tumors. Interestingly, survival analysis revealed a negative correlation between Cdr2 expression and patient survival in ccRCC patients. These findings provide evidence that Cdr2 might represent a novel important tumor antigen in kidney cancer and that its interaction with PHD1 could be involved the hypovascular morphology. Therefore, PHD1 expression was analyzed by immunohistochemical staining of tissue micro arrays (TMAs) of a wide number of RCC patients that have previously been analyzed for Cdr2 expression. PHD1 protein levels strongly correlated with the previously shown Cdr2 expression and was strong in pRCC. In addition, we developed enzyme-linked-immunosorbent-assays (ELISAs) to detect Cdr2 auto-antibodies as a diagnostic tool for cancer patients. While Cdr2 antibodies were present in the serum of paraneoplastic cerebellar degeneration patients, no Cdr2 antibodies were found in any of the analyzed pRCC or other RCC sera. This supports the fact that Cdr2 protein expression in cancer does not always correlate with the generation of Cdr2 auto-antibodies and the development of PCD.

## Zusammenfassung

Die vorliegende Dissertation ist in zwei Sektionen unterteilt. Im ersten Projekt wurde eine neue Rolle von Presenilin 1 und 2 (PSEN1/2) in der Regulierung der Hypoxie-Signalkaskade untersucht. Der zweite Teil befasst sich mit dem Protein Cdr2 (cerebellar degeneration-related protein 2) als potentieller Tumormarker für das papilläre Nierenzellkarzinom. Die beiden Projekte werden separat beschrieben und diskutiert mit einer allgemeinen Einführung über die Hypoxie zu Beginn.

Die Verfügbarkeit von Sauerstoff war eine der Grundvoraussetzungen für die Entwicklung von höherem Leben. Multizelluläre Organismen benötigen Sauerstoff, um effizient ATP zu produzieren. Deshalb hat die Evolution Mechanismen zur adäquaten Anpassung bei Sauerstoffmangel (Hypoxie) hervorgebracht. Unser Forschungsschwerpunkt liegt auf den molekularen und zellulären Mechanismen der Sauerstoffmessung.

Sauerstoffknappheit ist eine treibende Kraft für viele physiologische und pathophysiologische Prozesse, wie Höhenbergsteigen, die Bildung von roten Blutkörpern, Wundheilung, Krebs und auch neurodegenerativen Erkrankungen.

Die molekularen Sauerstoffsensoren einer Zelle sind Prolyl-4-hydroxylase Domänen enthaltende Proteine (PHDs), die die Degradation des Hypoxie-induzierbaren Faktors $\alpha$ (HIF$\alpha$) via sauerstoffabhängige Hydroxylierung regulieren. Unter normoxischen Bedingungen bindet der von Hippel-Lindau Tumor Suppressor (pVHL) an das hydroxylierte HIF$\alpha$, was zur poly-Ubiquitinierung und zum proteasomalen Abbau führt. In der Hypoxie ist der Kofaktor Sauerstoff limitiert und die Hydroxylierungsreaktion findet nur noch reduziert statt. Das stabilisierte HIF$\alpha$ translozíert in den Zellkern und bildet mit seinem konstitutiven Bindungspartner HIF$\beta$ einen potenten Transkriptionsfaktor. Der Transkriptionsfaktor HIF reguliert mehr als 200 Gene, die in der Anpassung an veränderten Sauerstoffbedingungen involviert sind. Es gibt zunehmend Hinweise darauf, dass neben der sauerstoffabhängigen Proteinregulation von HIF$\alpha$, pro-inflammatorische Stimuli wie Stickstoffmonoxid oder Zytokine HIF$\alpha$ auf der mRNA-Ebene regulieren.

*Projekt I:*

Wir konnten kürzlich zeigen, dass die Peptidyl-Prolyl *cis/trans* Isomerase FKBP38 spezifisch mit PHD2 interagiert und dessen Proteinstabilitität negativ reguliert (Barth et al.). Zudem wurde gezeigt, dass Presenilin 1 und 2 (PSEN1/2) an FKBP38 binden und dessen anti-apoptotische Wirkung verringern können. Die gleiche Gruppe fand, dass Presenilin-defiziente Maus embryonale Fibroblasten (MEFs) mehr FKBP38 Protein exprimieren als die wildtyp Kontrollen (Wang *et al.* 2005). Diese Publikation weckte unser Interesse an der Erforschung der Rolle der Preseniline in der PHD/HIF-Signalkaskade.

PSEN1/2 sind die Hauptkomponenten des γ-Sekretasekomplexes, der eine Vielzahl von Proteinen, wie das Amyloid Precursor Protein (APP) oder Notch, spaltet. Die Proteolyse von APP führt zur Bildung von Amyloid beta (Aβ), das in der Pathogenese der Alzheimers Erkrankung eine Rolle spielt. Gleichzeitig wird auch die Amyloid Precursor Intrazelluläre Domäne (AICD) gebildet, deren Funktion weitgehend unbekannt ist aber die in der Regulierung der Transkription involviert sein soll.

Wir konnten zeigen, dass PSEN1/2-defiziente MEFs und der Kortex von Vorderhirn-spezifischen konditionalen PSEN1/2 doppel knock-out Mäusen mehr FKBP38 Protein exprimieren und dass dies zu weniger PHD2 Protein führt. Während eine verringerte PHD2-Menge eine verstärkte hypoxische HIF-Akkumulierung erwarten lassen würde, fanden wir interessanterweise genau das Gegenteil. PSEN1/2 knock-out MEFs zeigten eine reduzierte HIF-Induktion in der Hypoxie, was auch zu einer reduzierten Expression der spezifischen HIF-abhängigen Zielgene führte. Während die HIF mRNA- und Proteinstabilität in den beiden Zelllinien gleich war, fanden wir weniger HIF-1α mRNA und eine verringerte HIF-1α Promotoraktivität in PSEN1/2 knock-out Zellen. Die proteolytische Funktion von PSEN1/2 war notwendig für eine normale HIF-Aktivierung. Faszinierenderweise beeinträchtigten die in Alzheimer Patienten beschriebenen Mutationen in PSEN1/2 die Hypoxieantwort auf unterschiedliche Weise. APP und die Generierung von AICD war entscheidend involviert in diesem Effekt. Zusammenfassend deuten unsere Daten auf eine direkte Rolle von PSEN1/2 über γ-Sekretase-abhängige Spaltung von APP/AICD in der Regulierung der Hypoxie Signalkaskade hin.

Bisher ist wenig über die biologische Funktion der Preseniline bekannt, so dass unsere Daten einen wichtigen Beitrag zu deren Verständnis leisten können. Mit steigender Lebenserwartung nimmt auch die Zahl der Alzheimerpatienten jährlich zu. Hypoxische Regionen im Gehirn korrelieren mit pathologischen Proteinablagerungen aber die Pathogenese ist weitgehend unbekannt. Unsere Resultate tragen deshalb zum Verständnis der Rolle von Hypoxie in der Pathologie von neurodegenerativen Erkrankungen bei.

*Projekt II:*

Neoplastische Expression des onconeuronalen Antigens Cdr2 is assoziiert mit cerebellären Degeneration in Brust- und Ovarkrebs. Die Cdr2 Protein Expression ist normalerweise auf Neuronen beschränkt, aber aberrante Cdr2 Expression in Tumoren kann zur Entwicklung einer Tumorimmunität führen, wobei das Krebswachstum beschränkt wird, was aber unglücklicherweise auch zu paraneoplastischer cerebellärer Degeneration führt. Bis jetzt wurde die Cdr2 Expression in Brust und Ovarkrebs beschrieben. Wir fanden eine starke Cdr2 Protein Exprimierung in 54.2 % der untersuchten papillären Nierenzellkrebs (pRCC) Tumoren im Vergleich zu einer viel niedrigeren Expression im klarzell Nierenzellkarzinom (ccRCC, 7.8%) (Balamurugan *et al.* 2009). Wir identifizierten PHD1 als neuen Cdr2 Interaktor und fanden eine erhöhte PHD1 Proteinmenge, wenn Cdr2 überexprimiert wurde. Die hohe PHD1 Proteinmenge korrelierten mit einer verminderten hypoxischen Antwort in Zelllinien wie auch in pRCC. Dies könnte eine Erklärung liefern, dass die pRCC die hypovaskulärste Nierenkrebsart ist. Interessanterweise korrelierte eine hohe Cdr2 Expression in ccRCC Patienten negativ mit ihren Überlebensraten. Diese Daten weisen auf eine wichtige neue Rolle von Cdr2 als prognostischer Marker für das Nierenzellkarzinom hin und die Interaktion mit Cdr2 könnte eine Erklärung für den hypovaskulären Phenotypen von pRCC liefern. Deshalb analysierten wir die PHD1 Expression in den zuvor mit Cdr2 gefärbten Tissue Microarray (TMA). Die PHD1 Proteinmenge war hoch in pRCC und korrelierte mit der zuvor gezeigten starken Cdr2 Färbung. Dies konnte im Immunoblot bestätigt werden. Die PHD1 Proteinexpression der kleineren, 40 kDa Isoform war in den pRCC höher als in den normalen Nieren. Zudem entwickelten wir enzymgekoppelte

Immunoadsorptionstest-assays (ELISAs) zur Detektierung von Cdr2 Antikörper in Patientenseren mit pRCC. Während Cdr2 Antikörper im Serum von Kontrollpatienten mit paraneoplastischer cerebellärer Degeneration detektiert wurden, konnten wir keine Cdr2 Antikörper in Seren von Patienten mit pRCC oder einem anderen RCC nachweisen. Da bisher keine pRCC-Patienten mit cerebellärer Degeneration beschrieben wurden, ist dieses Resultat auch nicht weiter erstaunlich. Zudem ist es mit dem Befund im Einklang, dass die aberrante Cdr2 Expression in Krebs nicht zwingend zu einer cerebellären Degeneration führen muss.

# General Introduction: Hypoxia

Free oxygen on Earth was not available for many billions of years before the first photosynthetic organisms evolved about 3 – 4 billion years ago (Pavlov and Kasting 2002). Initially, the oxygen in the oceans combined with dissolved iron and built banded iron formations. About 2.7 billion years ago, free oxygen started to outgas from the oceans and the period between 2.4 and 2.0 billion years has become generally known as the Great Oxygenation Event (GOE) (Barley *et al.* 2005). The availability of large amounts of free and dissolved oxygen may have allowed the aerobic organisms to produce more ATP than the anaerobic organisms, helping them to dominate Earth's atmosphere. The development of eukaryotic cells, complex multicellular organisms and ultimately plants and animals is highly intertwined with the evolution of photosynthesis and cellular respiration of oxygen.

Life has become highly dependent on oxygen and low oxygen partial pressures or hypoxia represent a major stress to cells and organisms. Hypoxia has many features and is involved in a wide range of physiological and pathophysiological processes such as adaptation to high altitude, glucose metabolism, wound healing as well as apoptosis, cancer and neurodegenerative processes. Scientific studies of adaptation to high altitude started in the 1860s when Viault counted his red blood cells before going and after adaptation to high altitude (Viault 1890). It then took almost 100 years until Allen Erslev discovered the responsible humoral factor, known today as erythropoietin (EPO) (Erslev 1953). A keystone in the elucidation of the molecular mechanism was the identification of the hypoxia inducible factor (HIF) by the discovery of a 3' flanking sequence in the erythropoietin gene that was sufficient for the expression of EPO under hypoxic conditions (Semenza and Wang 1992).

## 1 The Hypoxia-Inducible Factor (HIF)

### 1.1 Structure and Expression

One of the key oxygen sensors within the cell is the hypoxia-inducible transcription factor (HIF). HIF was purified in 1995 when Wang and Semenza characterized its hypoxic DNA binding activity (Wang and Semenza 1995). HIF is a heterodimeric

transcription factor consisting of an oxygen labile HIF-α subunit and an oxygen insensitive HIF-β subunit, also known as aryl hydrocarbon receptor nuclear translocator (ARNT). Both subunits are members of the basic helix-loop-helix Par/Arnt/Sim (bHLH-PAS) transcription factor family (Wang and Semenza 1995). In mammals, three HIF-isoforms have been found: HIF-1α, HIF-2α (also known as endothelial PAS domain protein (EPAS)1 or HIF-1α like factor (HLF)) and HIF-3α, all heterodimerizing with HIF-β. While HIF-1α is ubiquitously expressed in all tissues and organs, HIF-2α is primarily expressed in vascular endothelial cells, fibroblasts and epithelial cell lines (Tian *et al.* 1997; Wiesener *et al.* 1998) and HIF-3α is predominantly expressed in the kidney (Hara *et al.* 2001). HIF-α consists of an N-terminal bHLH domain which is essential of DNA binding and a PAS domain that is responsible for dimerization with ARNT (Jiang *et al.* 1996). The N-terminal and C-terminal oxygen-dependent degradation domains (NODDD and CODDD) are required for the oxygen-regulated degradation of HIF-α. The ODDD contains two conserved proline residues (P402 and P564 in HIF-1α). Additionally, two transactivation domains (TADs) are found within HIF-1α and 2α. The C-terminal domain is responsible for the recruitment of transcriptional co-activators and the expression of target genes (Jiang *et al.* 1996). HIF-α contains a C-terminal nuclear localization sequence (NLS).

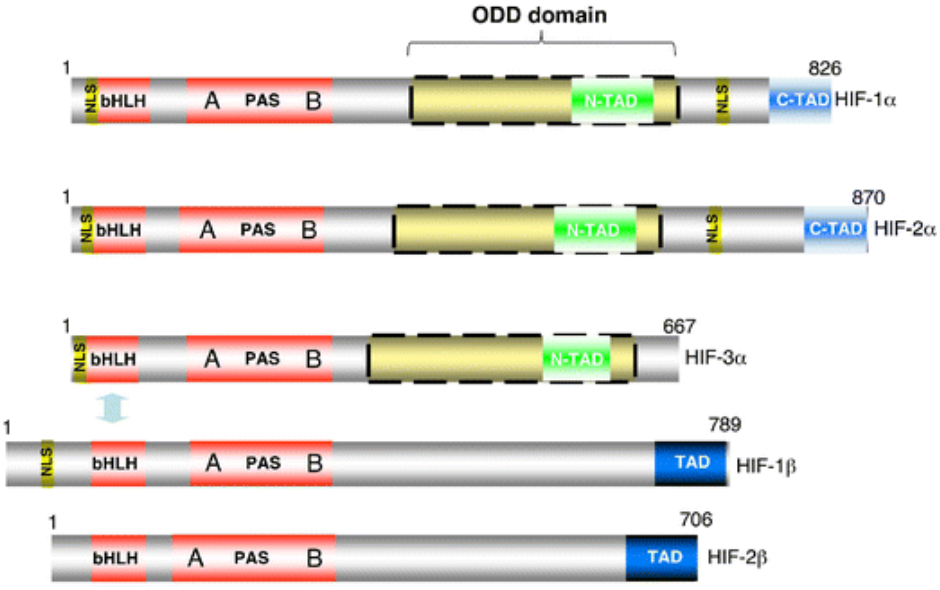


***Fig.1. Schematic representation of the HIF family member protein domains (Dayan et al. 2008).***

Genetic ablation of *HIF-1* results in embryonic lethality at day (E)11. *Hif1a-/-* embryos display developmental arrest with a defective neural tube, cardiovascular malformations, cell death within the cephalic mesenchyme and defective neural crest cell migration (Compernolle *et al.* 2003; Iyer *et al.* 1998). On the other hand, viable *Hif-2a*-deficient mice have been reported to have a syndrome of multiple-organ pathology and altered gene expression patterns together with enhanced generation of reactive oxygen species (ROS) (Scortegagna *et al.* 2003). Other studies revealed that *HIF-2a* knockout results in neonatal death proceeded by vascular disorganization, cardiac malfunction and insufficient production of surfactant that is likely to be fatal (Compernolle *et al.* 2002; Peng *et al.* 2000; Tian *et al.* 1998). Genetic ablation of the HIF-3$\alpha$ subunit in mice has not yet been reported.

## 1.2 Regulation

### *1.2.1 Oxygen-dependent Regulation of HIF$\alpha$ Protein Stability*

The HIF-$\alpha$ protein stability, but not HIF-$\alpha$ mRNA expression, is tightly and directly regulated by the availability of oxygen. HIF-$\alpha$ subunits undergo rapid oxygen-dependent degradation via the ubiquitin-proteasome pathway (Kallio *et al.* 1999; Salceda and Caro 1997). In normoxia, two prolines (HIF-1$\alpha$: 402 and 564) within the ODD of the human HIF-$\alpha$ protein are hydroxylated by specific HIF prolyl-4-hydroxylases (PHDs). This hydroxylation is a prerequisite for binding of the von Hippel Lindau pVHL tumor suppressor protein complex (Maxwell *et al.* 1999) and subsequent destruction by the 26 proteasome. When oxygen becomes limiting, the prolyl hydroxylation reaction is inhibited and the stabilized HIF-$\alpha$ translocates to the nucleus where it heterodimerizes with HIF-$\beta$ (Kallio, *et al*. 1998; Jewell *et al.* 2001). Normoxic HIF-$\alpha$ stabilization due to loss of pVHL was documented in vascular tumors like clear cell renal cell carcinoma (ccRCC), hemangioblastoma and pheochromocytoma (Maxwell *et al.* 1999). The heterodimeric HIF transcription factor binds to hypoxia responsive elements (HRE) within the promoter and enhancer region of its target genes (Wenger 2002).

In addition to prolyl hydroxylation, HIF-$\alpha$ is hydroxylated in oxygenated cells at a specific asparagine residue (hHIF-1$\alpha$: Asn803) by another hydroxylase named factor inhibiting HIF (FIH) (Lando *et al.* 2002; Mahon *et al.* 2001). This hydroxylation blocks the

interaction of HIF with the coactivators p300 and CBP (Lando *et al.* 2002). Like the PHDs, FIH activity is inhibited under hypoxia, allowing HIF to act as a powerful transcription factor.

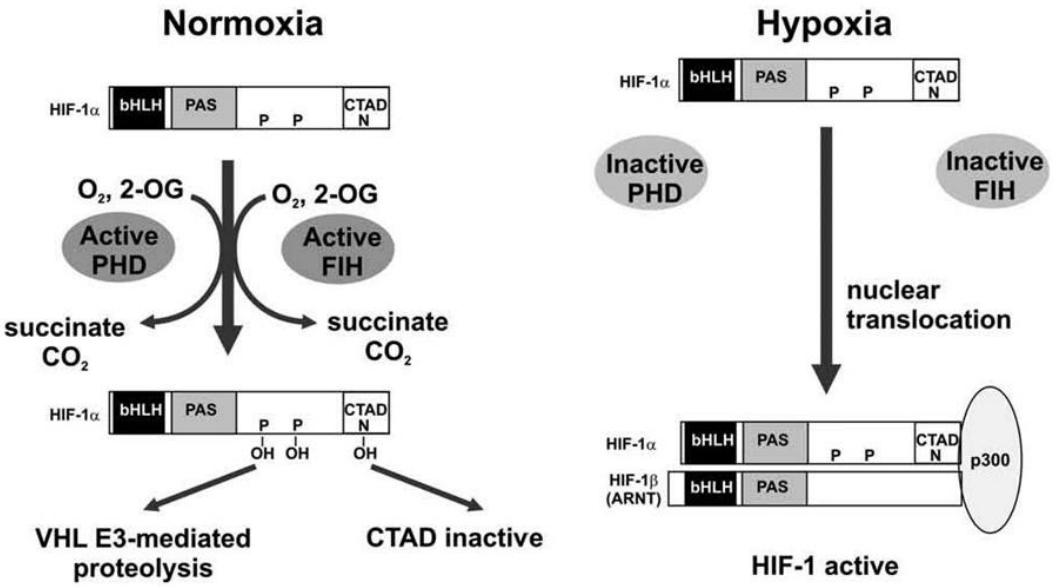


***Fig.2. Oxygen-dependent regulation of HIF-1α (Metzen and Ratcliffe 2004)*** *Under normoxia, HIF-1α is hydroxylated on two conserved prolines which leads to the recognition by pVHL and ubiquitin-mediated proteasomal degradation. The availability of oxygen also results in hydroxylation of an asparagine residue by FIH which inactivates the C-terminal transactivation domain. Since the hydroxylase activity is inhibited in hypoxia, HIF-1α is stabilized, translocates to the nucleus where HIF-1β and p300/CBP are recruited to activate the transcription of its target genes.*

### *1.2.2 Oxygen-independent Regulation*

Besides the oxygen-dependent post-translational regulation of HIF-α, a number of other mechanisms contribute to the regulation of HIF. S-nitrosylation of the cysteine residue 800 (C800) has been reported to activate the interaction between stabilized HIF-1α and the co-activator CBP/300, eventually stimulating the transactivation of the HIF-complex (Yasinska and Sumbayev 2003). Another mechanism that has been shown to increase HIF transcriptional activity is via the phosphorylation of threonine 796 and of the serine 641 and 643 by the mitogen-activated protein kinases (MAPKs) (Lancaster *et al.* 2004; Richard *et al.* 1999; Sang *et al.* 2003). Conflicting results about SUMOylation increasing or decreasing HIF-α stability have been reported (Bae *et al.* 2004; Berta *et al.* 2007; Carbia-Nagashima *et al.* 2007; Cheng *et al.* 2007). A recent study showed that

sumoylation increases HIF-1α stability, thereby improving brain stem cardiovascular regulation in experimental rat models of brain death (Chan *et al.* 2011). There is growing evidence that hormones and cytokines like IL1-β, TNA-α as well as ROS and LPS can regulate normoxic HIF-1α mRNA levels via a conserved NFκB site in the *HIF1a* promoter (Bonello *et al.* 2007; Frede *et al.* 2006; Gonzalez-Perez *et al.* 2010; Görlach 2009; Qian *et al.* 2004). A variety of oncoproteins, and growth factors have been shown to regulate normoxic HIF-1α synthesis via the activation of the phosphatidylinositol 3-kinase (PI3K) or mTOR pathways (Fukuda *et al.* 2002; Zhong *et al.* 2000), reviewed in (Semenza 2003).

Furthermore, the inhibitory PAS domain protein (IPAS), an alternative splice variant of HIF-3α, acts as a dominant negative transcription factor that represses HIF-α activity (Makino *et al.* 2001).

## 2 The Prolyl-4-Hydroxylases

The identification and characterization of the first HIF prolyl-4-hydroxylase resulted from work in *C. elegans* where it was named egg-laying abnormal 9 (EGL-9) (Epstein *et al.* 2001). Three ortholog isoforms could be identified in humans and were named PHD1 (alternatively termed egl nine homolog EGLN2 or HIF prolyl-hydroxylase (HPH) 3), PHD2 (EGLN1, HPH2), and PHD3 (EGLN3, HPH1) (Bruick and McKnight 2001; Ivan *et al.* 2002). In addition, a fourth PHD-related protein (PH-4) with an endoplasmic reticulum transmembrane domain has been identified (Koivunen *et al.* 2007; Oehme *et al.* 2002). PH-4 is capable of hydroxylating HIF-α *in vitro* and in cultured cells but it remains to be established whether it also participates in the regulation of HIF-α *in vivo* .

### 2.1 PHD1

PHD1 is located on chromosome 19q13.2 and encodes a 407 AA protein. Its mRNA is ubiquitously expressed but most abundant in testis (Lieb *et al.* 2002). GFP-tagged PHD1 overexpression experiments suggested that PHD1 is mainly localized in the nucleus (Metzen *et al.* 2003). Other studies using monoclonal PHD1 antibodies showed predominantly cytoplasmic localization (Soilleux *et al.* 2005). Homozygous knock out of PHD1 facilitated HIF-1α-mediated cardio-protection in ischemia/reperfused myocardium

and protected against the development of colitis though reduced epithelial apoptosis and increased barrier function (Adluri *et al.* 2011; Tambuwala *et al.* 2010) (Schneider *et al.* 2010). Furthermore, genetic ablation and inhibition of PHD1 was shown to induce hypoxia tolerance by reprogramming basal metabolism and therefore protecting myofibres against lethal ischemia (Aragonés *et al.* 2008).

### 2.2 PHD2

The PHD2 gene is located on chromosome 1q42-43 and encodes a 426 AA protein. Its mRNA is ubiquitously and abundantly expressed (Lieb *et al.* 2002). In non-malignant tissue, PHD2 is mainly localized in the cytoplasm but increases nuclear expression was observed in less differentiated types of cancer cells (Jokilehto *et al.* 2006). Since only PHD2, but not PHD1 or PHD3, knock out embryos die between E12.5 and E14.5, PHD2 is often regarded as the main regulator of HIF (Minamishima *et al.* 2008; Takeda *et al.* 2008). Mutations in the PHD2 gene have been demonstrated to cause erythrocytosis, a familial hereditary disease characterized by excessive production of erythrocytes (Ladroue *et al.* 2012; Percy *et al.* 2006)

### 2.3 PHD3

The PHD3 gene is located on chromosome 14q13.1 and encodes a 239 AA protein. Its mRNA is ubiquitously expressed and particularly abundant in heart and liver (Lieb *et al.* 2002). PHD3 was shown to be localized both in the cytoplasm and the nucleus (Soilleux *et al.* 2005). PHD3 knock-out mice show reduced neuronal apoptosis, abnormal sympathoadrenal development and reduced blood pressure (Bishop *et al.* 2008). Addionally PHD3 was shown to be essential for hypoxic regulation of neutrophilic inflammation and that loss of PHD3 enhances the innate immune response to abdominal sepsis (Kiss *et al.* 2012; Walmsley *et al.* 2011).

### 2.4 PHD-interacting Proteins

Screening for novel PHD interacting proteins, a number of candidates have been identified in the last years that affect PHD stability, enzymatic activity and function in different ways. Characterized PHD interacting proteins are listed in Fig. 3 (Wenger *et al.* 2009).

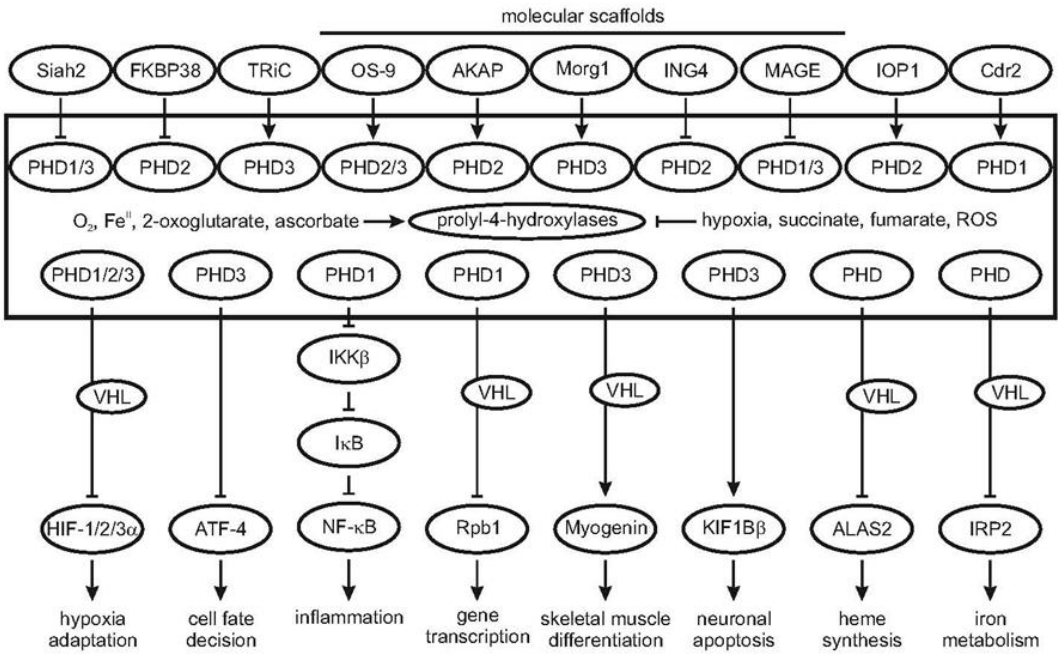


***Fig.3. Simplified schematic overview on the PHD interacting proteins and their function (Wenger et al. 2009).***

In our previous studies, we found the FK506-binding protein FKBP38 to specifically interact with PHD2 and to regulate PHD2 protein stability (Barth *et al.* 2009; Barth *et al.* 2007). Furthermore, we showed that the cerebellar degeneration-related antigen Cdr2 interacts PHD1 and leads to a blunted hypoxic response (Balamurugan *et al.* 2009).
This thesis is based on this previous work from our group and was divided into two projects that will be discussed separately.

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## 4 Aims of the Thesis

*Project I*

The first aim of this thesis was to investigate the role of presenilin 1 and presenilin 2 in the regulation of the PHD/HIF signaling pathway.

*Project II*

The aim of the second project was to characterize Cdr2 as a potential marker for papillary renal cell carcinoma.

# Project I: Presenilin Function in the Oxygen Sensing Pathway

## 1 Introduction

### 1.1 FKBP38

#### *1.1.1 Discovery and Structure*

The FK506binding protein 38 (FKBP38) is a member of the FKBP family that binds the immunosuppressive drug FK506. FK506-binding proteins belong to the superfamily of PPIases together with cyclophilins (Cyps), parvulins and the recently found protein serine/threonine phosphatase 2a (PP2A) (Barik 2006). The name FK506 comes from the catalogue number of Fermentek, the company that synthesized Tacrolimus, a macrolid lactone of the gram-positive bacteria *Streptomyces tsukubaensis* that was discovered in 1984. FKBP38 was first described in 1995 by (Lam *et al.* 1995). Novel FKBP homologs were identified by screening Jurkat cDNAs that contain the DNA sequence for the well conserved second β-sheet region of FKBP12 from AA26 to 34 (Lam *et al.* 1995). A cDNA was found that encodes a 38 kDa protein that is 33% identical to FKBP12. It later turned out that the 38 kDa resulted from a truncated open reading frame. A 47 AA encoded extended open reading frame of FKBP38 was found and named FKBP8 (Nielsen *et al.* 2004).

The human FKBP38 gene is located on chromosome 19p12. FKBP38 contains a PPIase domain, three 34 AA long tetratricopeptide repeat domains (TPRs) similar to FKBP52, a CaM binding site and a transmembrane domain (TM) at the C-terminus. FKBP38 is an integral membrane protein located in the mitochondria and ER

membranes exposed to the cytosolic side. FKBP38 was suggested to be cell type specifically posttranslationally modified during development (Bulgakov *et al.* 2004).


***Fig.1. FKBP38 protein structure.*** *Schematic diagram of functional domains. Other FKBP38.interacting domains are also shown. FKBD = FK binding domain, TRP = tetratricopeptide repeat domains, CBD = calmodulin binding domain, TM = transmembrane domain (Kang et al. 2008).*

FKBP38 is ubiquitously expressed with highest levels in brain, followed by moderate expression in the heart, lung, skeletal muscle and pancreas. FKBP38 protein is widely expressed in murine embryonic and adult tissues as well as in human cancer cell lines (Bulgakov *et al.* 2004; Kang *et al.* 2005).

FKBP38 is distinct from its other family members as it lacks the conserved amino acid tryptophan (W) at position 59 and has instead a Leucin (L). Tryptophan forms the basis of the hydrophobic FK506 drug-binding cavity. It is therefore thought that FKBP38 does not contain a PPIase activity (Lam *et al.* 1995). FKBP38 alone does not show *in vitro cis/trans* isomerase activity. However, it absolutely requires $Ca^{2+}$ and CaM as co-factors to function as an active enzyme (Edlich *et al.* 2005).

Genetic disruption of FKBP38 resulted in embryonic lethality at embryonic day E13.5 (Bulgakov *et al.* 2004). Detailed analysis revealed defects in the development of the central nervous system. The phenotype seems to comparable to constitutively active sonic hedgehog signaling pathway (SHH) or inhibition of the SHH antagonist protein kinase A (PKA) (Perron *et al.* 2003).

### *1.1.2 Functions and Regulation of FKBP38*

FKBP38 was shown to have multiple functions in cell regulatory processes such as cell signaling, apoptosis, cell growth and proliferation.

FKBP38 was proposed to play a role in apoptosis by binding to the anti-apoptotic protein Bcl-2 but its mechanism and relevance are still under debate (Shirane and

Nakayama, 2003). A recent study demonstrated that the binding of FKBP38 to Bcl-2 is mainly mediated by the charge-sensitive loop near the putative active site of FKBP38 (Haupt *et al.* 2012). In HeLa cells, FKBP38 targets Bcl-2 as well as Bcl-$x_L$ to mitochondrial membranes thereby facilitating Bcl-2 and Bcl-$x_L$ in their antiapoptotic role (Shirane and Nakayama 2003). FKBP38 downregulation by RNAi strongly reduced HeLa cell viability and greatly enhanced caspase-3 activity as well as PARP cleavage (Kang *et al.* 2005). FKBP38 has been shown to play a role in the mTOR pathway by competing with Rheb for mTOR binding (Bai *et al.* 2007; Rosner *et al.* 2003). Under conditions of sufficient nutrients, GTP-Rheb was shown to accumulate and prevent FKBP38 binding to mTOR and activate the phosphorylation of the mTOR targets S6K and 4E-BP. Therefore, FKBP38 might function as an endogenous mTOR inhibitor by interfering with Rheb and modulating cell growth and size. Overexpression of FKBP38 has been observed in various human cancer cells and tumor tissues, such as breast, colon, liver, lung, lymph node, prostate and stomach cancers (Choi *et al.* 2010) (Kang *et al.* 2005). Furthermore, Edlich *et al.* reported a $Ca^{2+}$/CaM dependent interaction of FKBP38 with Bcl-2 that is blocked by Hsp90 binding to FKBP38. However, they propose that FKBP38 promotes cell death in SH-SY5Y neuroblastoma cells (Edlich *et al.* 2005; Erdmann *et al.* 2007). FKBP38 was shown to have an intrinsic inhibitory effect on calcineurin (Shirane and Nakayama 2003). Tagged isolated FKBP38 from HeLa cells reduced the dephosphorylation capacity of calcineurin on synthetic phosphopeptide of RII subunit of PKA. Contrary to Shirane *et al*., other groups did not observe the inhibitory effect on calcineurin phosphatase activity by FKBP38 *in vitro* (Kang *et al.* 2005; Weiwad *et al.* 2005). More recently, FKBP38 was proposed t promote the folding of cystic fibrosis transmembrane conductance regulator (CFTR) in the ER and therefore to play a role in membrane protein biogenesis (Banasavadi-Siddegowda *et al.* 2011).

We previously identified FKBP38 to specifically interact with PHD2 and to negatively regulate its protein stability (Barth *et al.* 2009; Barth *et al.* 2007). Protein stability of FKBP38 and Bcl-2 was shown to be determined by presenilin 1 and 2 (PSEN1 and PSEN2) and genetic ablation of PSEN1/2 increased FKBP38 and Bcl-2 protein abundance, rendering cells less susceptible to apoptotic stimuli (Wang *et al.* 2005).

## 1.2 Presenilins

### *1.2.1 Structure, Expression and Function*

Presenilins (PSEN) were discovered through their linkage to early onset familial Alzheimer's disease (FAD) in 1995 (Sherrington *et al.* 1995). PSEN1 is localized on chromosome 14q24.3 and PSEN2 on 1q31-q42. The PSEN1 gene was found by direct cDNA selection of yeast artificial chromosomes comprising the putative chromosomal region. Subsequently, the PSEN2 gene was identified due to its high sequence homology with PSEN1 and its location with the candidate regions was defined in AD linkage studies. Two years after its discovery, PSEN1 was shown to be essential for the generation of the Notch intracellular domain (NICD) in *C. elegans* (Wong *et al.* 1997). Subsequent research elucidated the function of PSEN as the catalytic subunit of the γ-secretase complex (reviewed in De Strooper and Annaert 2010).

PSEN 1 and 2 contain 9 transmembrane domains and are cleaved in a N-terminal (27-30 kDa) and C-terminal (16-18 kDa) fragment that remain associated with the complex (Henricson *et al.* 2005). Whereas PSEN1 is uniformly expressed throughout the brain and in peripheral tissues, PSEN2 expression in the brain is relatively low, except in the corpus collosum. PSEN2 is highly expressed in some peripheral tissues, such as pancreas, heart and skeletal muscle (Rogaev *et al.* 1997). PSEN1 knockout is embryonically lethal and resembles the Notch knockout phenotype, indicating its essential role during development (De Strooper *et al.* 1998; Shen *et al.* 1997; Wong *et al.* 1997). PSEN2 ablation leads to a mild pulmonary phenotype and no changes in amyloid precursor protein (APP) processing suggesting that PSEN1 and 2 have at least partially overlapping functions *in vivo* but that PSEN1 is essential for normal Notch signaling (Herreman *et al.* 1999). Several groups made $PSEN1^{+/-}/PSEN2^{-/-}$ or forebrain-specific conditional knockout mice to study the role of PSEN in long term development and brain function (Donoviel *et al.* 1999; Saura *et al.* 2004). These studies have shown that besides the central nervous system, the skin (Soriano *et al.* 2001; Xia *et al.* 2001) and the immune system (Beglopoulos *et al.* 2004; Tournoy *et al.* 2004) are severely affected by the loss of PSEN1/2.

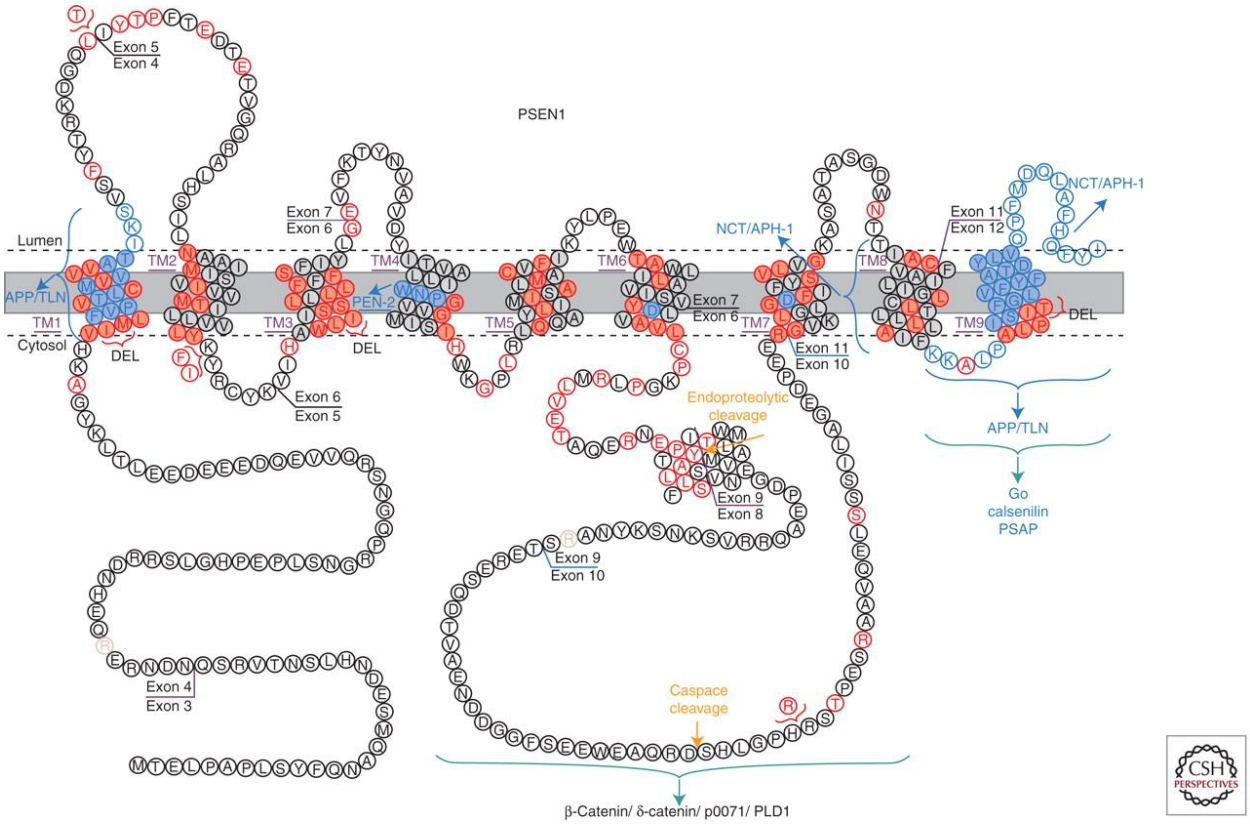


***Fig.2. Presenilin nine-transmembrane domain structure.*** *A view on the reported PSEN1 mutations (red) and the nine-transmembrane domain structure. Drawing by R. Crook (Hardy 2007)*

### *1.2.2 Presenilins and the γ-Secretase Complex*

The γ-secretase complex is a intramembrane multisubunit protease consisting of either PSEN1 or PSEN2, presenilin enhancer 2 (Pen-2), nicastrin (NCT), anterior pharynx 1 (Aph-1) that cleaves many type I transmembrane proteins (De Strooper *et al.* 1999; Parks and Curtis 2007). Pen-2 is a small hairpin-like protein with a molecular weight of ~12 kDa (Francis *et al.* 2002). NCT is a 130 kDa type I integral membrane glycoprotein and Aph-1 is a ~25 kDa protein with 7 TM-domains (Goutte 2002; Yu *et al.* 2000). Extensive reconstitution studies in mammalian and yeast cells have demonstrated that all four proteins are necessary and sufficient for the generation of an active γ-secretase (Edbauer *et al.* 2003; Kimberly *et al.* 2003). The γ-secretase belongs to an unique group of intramembrane-cleaving proteases (I-CLiPs) (Wolfe and Kopan 2004). Electron microscopy studies revealed that the γ-secretase complex forms a large spherical structure containing two small central cavities with opposite orientations (Lazarov *et al.* 2006). These small extracellular and cytoplasmic openings were proposed to be exit

sites for cleavage products from the water-containing cavity. Supporting these findings, succeeding studies demonstrated that the active-site region in PSEN's TMD6 and TMD7 are water accessible (Sato *et al.* 2006; Tolia *et al.* 2006) and that the TMD9 and the hydrophobic domain in the large cytoplasmic loop of PSEN are dynamic parts of the water-containing cavity (Tolia *et al.* 2008). A recent study showed that the first hydrophobic region in TMD1 is facing the catalytic pore, indicating a functional role in the catalytic pore structure (Takagi *et al.* 2010).

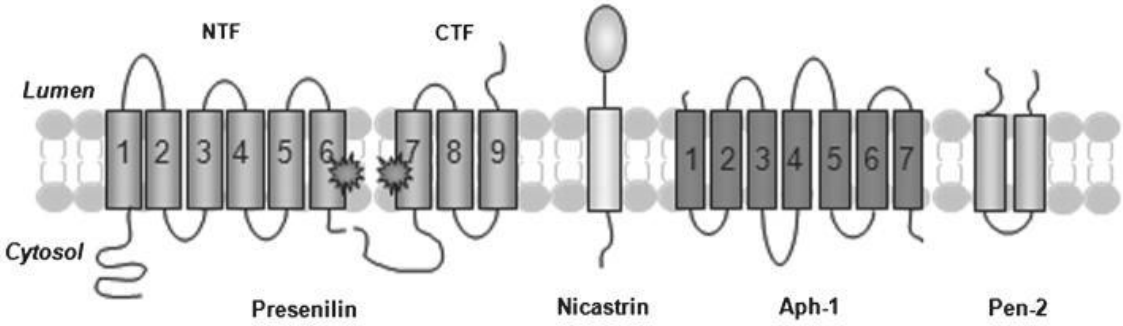


***Fig.3. PSEN/γ-secretase complex (Haapasalo and Kovacs 2011).*** *The γ-secretase consists of four components, PSEN 1 or 2, Nicastrin, Aph-1 and Pen-2.*

The most prominent targets of the γ-secretase are the amyloid-β (Aβ) precursor protein (APP) and Notch but to date, more than 90 substrates have been described (Haapasalo and Kovacs 2011). Some of the well-known substrates are listed in table 1 and are extensively described in (Haapasalo and Kovacs 2011).

| **Substrate** | **Function** | **γ-secretase product** |
|---|---|---|
| Alcadein α | Regulation of AβPP signaling and processing | 1. β-Alc<br>2. Alc-ICD |
| APLP1 | Synaptogenesis | 1. ALID1<br>2. p3-like fragment |
| APLP2 | Neurite outgrowth | 1. ALID2<br>2. p3-like fragment<br>3. Aβ-like fragment |
| ApoER2 | Lipid metabolism | ApoER2ICD |
| APP | Cell adhesion, neurite outgrowth, protein transport (?) | 1. Aβ<br>2. AICD<br>3. p3 |
| CD44 | Cell adhesion, hyaluronan receptor | 1. CD44-ICD<br>2. CD44-β |

| | | |
|---|---|---|
| Delta1 | Notch ligand, cell fate determination | DICD |
| E-Cadherin | Cell adhesion | E-Cad/CTF2 |
| EphrinB1 | Cell-cell adhesion | eB1ICD |
| EphrinB2 | Axon guidance | ephrinB2-CTF2 |
| ErbB4 | Cell proliferation, differentiation, apoptosis, myelination | E4ICD |
| IGF-1R | Insulin-like growth factor 1 receptor | IGF-1R-ICD |
| Jagged 2 | Notch ligand, cell fate determination | JICD |
| N-Cadherin | Cell adhesion, synapse formation and maintenance | N-cad/CTF2 |
| Neuregulin-1 | ErbB receptor ligand, regulation of Schwann cell proliferation | NRG1-ICD |
| Notch 1-4 | Signaling receptor, cell fate determination | NICD |
| $p75^{NTR}$ | Neurotrophin receptor, cell survival/death, cell migration, axon guidance | p75-ICD |
| Syndecan 1-3 | HSP, neurite outgrowth, cell migration, learning, memory | SICD/ n.d. |
| VEGF-R1 | Angiogenesis | VEGF-R1-ICD |

***Table 1. PSEN/γ-secretase substrates and the proposed roles of their cleavage products (modified from (Haapasalo and Kovacs 2011).***

### *1.2.3 Amyloid Precursor Protein Processing*

The APP gene is located on chromosome 21 and encodes a 770 AA protein (Goldgaber *et al.* 1987). APP is a single pass type I transmembrane protein that undergoes sequential cleavage via two different pathways with functionally different outcomes (De Strooper and Annaert 2000). The primary cleavage occurs by either the α- or the β-secretase and leads to the generation of the extracellular domain (sAPP). Subsequent intramembrane processing of the remaining membrane tethered C-terminal fragment by the γ-secretase complex releases the APP intracellular domain (AICD). Initial cleavage by α-secretase occurs within the Aβ domain, generating the secreted sAPPα fragment with neurotrophic properties and thus preventing the generation of toxic Aβ peptide (Hardy 1992). Therefore, it is also referred to as the “non-amyloidogenic pathway” (Lammich *et al.* 1999). Sequential cleavage by β- and γ-secretase releases the Aβ peptide that has neurotoxic properties and is believed to be central to AD as postulated in the amyloid cascade hypothesis (Hardy and Selkoe 2002).

The physiological function of APP is incompletely understood but it has been shown to be important in early development, such as mediating cell adhesion, cell migration and synaptogenesis (reviewed in Guo *et al.* 2012). APP ko mice are viable and fertile despite reduced body and brain weight (Müller *et al.* 1994), increased levels of copper and iron in the liver and cerebral cortex (Duce *et al.* 2010; White *et al.* 1999) and suffer from lower forelimb grip strength, impaired locomotor activity and passive learning avoidance (Dawson *et al.* 1999; Senechal *et al.* 2008).

While the function of Aβ in the pathogenesis of AD is widely studied, the role of AICD is less well understood. Since AICD was discovered only 23 years after the identification of APP (Ohsawa *et al.* 1999) and it is rapidly degraded after release from the membrane (Cupers *et al.* 2001; Edbauer *et al.* 2002), research on AICD is lagging behind. AICD has been proposed to be involved in transcriptional regulation, in a manner analogous to Notch signaling. The first hint that AICD might be involved in transcriptional regulation came from Gal4-DBD-AICD fusion construct experiments in yeast (Cao and Sudhof 2001). In subsequent studies, several genes regulated by AICD have been identified, including KAI1 (Baek *et al.* 2002), glycogen synthase 3β (Kim *et al.* 2003), Neprilysin (Belyaev *et al.* 2009) APP and BACE (von Rotz *et al.* 2004), cyclins B1 and D1 (Ahn *et al.* 2008), the vesicular glutamate transporter VGLUT2 (Schrenk-Siemens *et al.* 2008) and C/ebP homologous protein (CHOP) (Takahashi *et al.* 2009). Addionally, EGF receptor and LRP1 were described to be downregulated by AICD (Liu *et al.* 2007; Zhang *et al.* 2007).

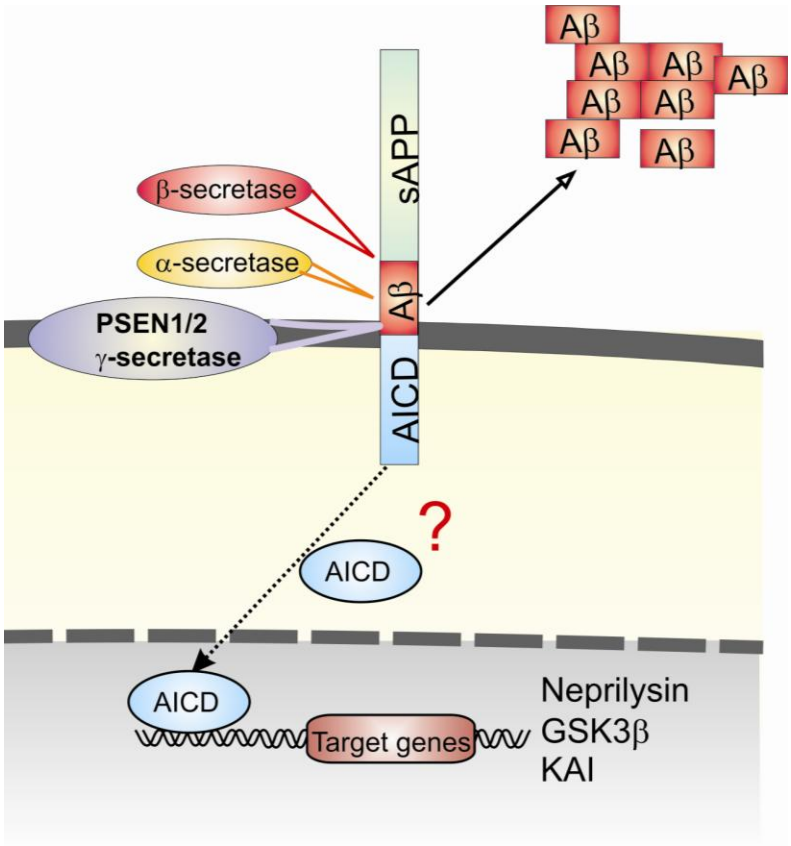


***Fig.4. Simplified schematic presentation of APP processing.*** *The first cleavage occurs by either the α- or the β-secretase that releases the extracellular domain (sAPP). Subsequent processing by the γ-secretase. leads to the generation of the amyloid precursor intracellular domain (AICD). Initial α–secretase cleavage occurs with the Aβ domain, generating the sAPPa fragment. Cleavage by the β and γ-secretase leads to the generation of the neurotoxic Aβ peptide.*

### *1.2.4 Notch and Other γ-Secretase Substrates*

Besides the growing list of proteins that are cleaved by the γ-secretase, the Notch receptors (Notch 1-4) are amongst of the most well known and important substrates of the γ-secretase (De Strooper *et al.* 1999; Donoviel *et al.* 1999; Herreman *et al.* 2000; Wong *et al.* 1997). Notch is an evolutionary conserved transmembrane receptor that is involved in the regulation of many developmental aspects such as cell fate and stem cell maintenance. Notch functions at the cell surface and mediates cell-cell signaling interactions to specify cell fates during development (Bray 2006).

The dysregulation of the Notch pathway is involved in a wide range of human disorders and cancer progression and Notch inhibition is a promising approach for cancer treatment (Purow 2012).

The Notch receptor is activated by binding to a ligand presented by a neighboring cell. Upon ligand binding, Notch gets cleaved by a membrane tethered metalloprotease (ADAM). Subsequent γ-secretase cleavage releases the Notch intracellular domain (NICD) and Nβpeptide (De Strooper *et al.* 1999). NICD then translocates to the nucleus where it associates with the DNA-binding protein CSL (CBF1/FBPjk/Su(H)/Lag-1) (Schroeter *et al.* 1998). Upon NICD binding, allosteric changes occur in CSL that facilitate displacement of transcriptional repressors and the recruitment of co-activators that leads to the transcription of target genes. PSEN1 knockout or PSEN1/2 double knockout mice have defects reminiscent of Notch knockout phenotypes (De Strooper *et al.* 1998; Shen *et al.* 1997; Wong *et al.* 1997).

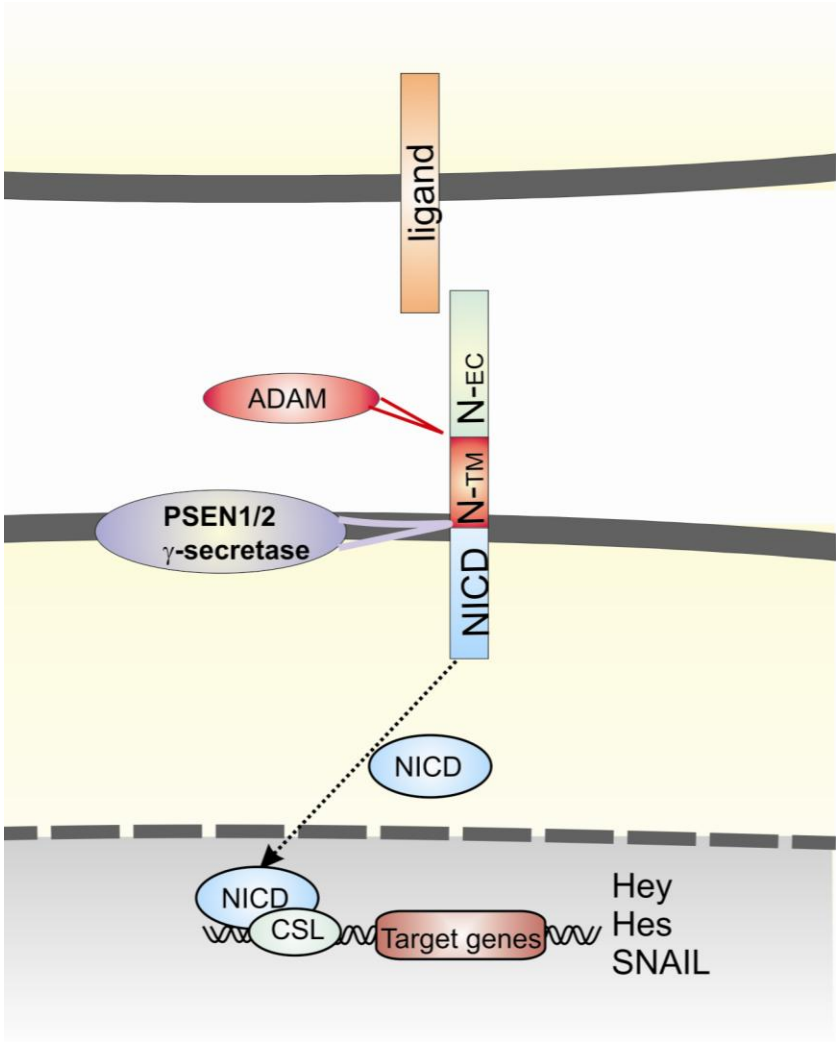


***Fig.5. Simplified schematic presentation of Notch processing.*** *Upon Notch ligand binding, ADAM-type metalloprotease and subsequent intramembrane γ-secretase cleavages release the Notch intracellular domain (NICD). NICD drives the expression of its target genes such as Hey, Hes and SNAIL.*

### 1.2.5 γ-Secretase Independent Functions of Presenilins

PSENs were described to have γ-secretase independent functions but it is difficult to provide strong evidence since genetic knock-out models of PSEN and pharmacological inactivation of γ-secretase are difficult to compare *in vivo* (Wakabayashi *et al.* 2008). The described γ-secretase independent functions rely mostly on its ability to interact with other proteins. PSEN was found in complex with β-catenin and other members of the armadillo family (Zhang *et al.* 1998; Zhou *et al.* 1997). Some of the most notable γ-secretase independent roles of PSEN are the regulation of calcium homeostasis, insulin and PI3K signaling (Maesako *et al.* 2011) reviewed in (LaFerla 2002). The first description of a $Ca^{2+}$ defects in fibroblasts from AD patients dates back to 1994, even before the discovery of PSEN (Ito *et al.* 1994). Subsequent work clearly showed that PSENs are involved in $Ca^{2+}$ homeostasis but full mechanistic insight is still missing (Bezprozvanny and Mattson 2008; De Strooper and Annaert 2010).

PSEN-deficiency has been shown to lead to a sorting defect of intracellular adhesion molecule 5 (ICAM 5) or telecephalin (Esselens *et al.* 2004), epidermal growth factor receptor (EGFR) (Repetto *et al.* 2007) and β1 integrin (Zou *et al.* 2008). More recently, PSEN1 was suggested to be involved in the maturation of the V0a subunit and subsequent assembly of the V-APTase proton pump (Lee *et al.* 2010). Further evidence that PSEN might be involved in membrane trafficking comes from a report that shows that several members of the Rab family of small GTPases bind to PSEN (Dumanchin *et al.* 1999).

### 1.2.6 Presenilins in Alzheimer's Disease

Alzheimer's disease is the world's most common neurodegenerative disease (Selkoe 2001). It is estimated that about 18 million people worldwide are currently suffering from AD. While the molecular mechanism of disease progression is not fully understood, the main risk factor of AD is ageing. With increasing life expectancy, the number of patients is predicted to be double by 2025.

### *Histopathology of Alzheimer's Disease*

In 1906, the German psychiatrist Alois Alzheimer was the first to describe the symptoms of a disease that was later named after him. One of his patients suffered from "progressive memory impairment, disordered cognitive function, altered behavior including paranoia, delusions, loss of social appropriateness and a progressive decline in language function" – symptoms that are still relevant for diagnosis more than 100 years later. Postmortem analysis of the brain of the first AD patient, Auguste D revealed cerebral atrophy, "many fibrils" in the cell and "military foci" all over the cortex.. The lesions that Alzheimer reported are still the pathological hallmarks of AD and are described as neuronal cell loss, intracellular accumulation of neurofibrillary tangles and the extracellular accumulation of senile plaques. The senile plaques are composed of the 4 kDa $A\beta_{40}$ and the more amyloidogenic $A\beta_{42}$, highly hydrophobic peptides that aggregates to form oligomers (for review see Hardy *et al.* 2002).

### *Genetics of Alzheimer's Disease*

5 – 10% of AD cases are genetically inherited as familial AD (FAD) while the majority of the cases are sporadic (Selkoe 2001). Most FAD cases are caused by mutations in the PSEN1 gene whereas mutations in the amyloid precursor protein (APP) and PSEN2 are rather rare. To date, more than 170 coding single nucleotide polymorphisms (SNPs) in PSEN1 have been identified in 370 AD families, with onset ranging between 16 and 65 years of age. SNPs in PSEN2 are variably penetrant and rare, despite significant structural similarities with PSEN1. Only 10 PSEN2 mutations in 18 AD families have been reported (www.molgen.ua-ac.be/ADMutations). Most of the known mutations cause amino acid substitution and result in the relative increase in the ratio of the β-amyloid 42 $(A\beta)_{42}$ to $A\beta_{40}$ peptides (Scheuner *et al.* 1996). Since this can be caused by either an increase in $A\beta_{42}$ or a decrease in $A\beta_{40}$, there is an ongoing debate whether clinical mutations cause a "gain of function" or rather a "loss of function" γ-secretase activity (Bertram *et al.* 2010). Bentahir *et al* significantly contributed to the understanding of altered γ-secretase function of PSEN mutations by expressing clinical relevant mutants in PSEN deficient cells (Bentahir *et al.* 2006). Their study revealed a (partial) loss of function in all investigated clinical mutants. This was the consequence of

either lowering $A\beta_{40}$ or increasing $A\beta_{42}$ or both. The cleavage of other analyzed $\gamma$-secretase substrates such as Notch, syndecan and N-cadherin were differentially affected by the clinical mutations.

In contrary to the cases of FAD, the reasons for developing sporadic AD are unclear. It is discussed that the balance between generation and clearance of $A\beta$ is altered by aging but further investigations are required the cause of sporadic AD. Whether $A\beta$ clearance is sufficient or clinical benefits over time is currently evaluated in AD patients who received active vaccination (Holmes *et al*., 2008).

## 2 Working Hypothesis

A recent publication showed that PSEN1/2 interact with FKBP38 and hence promote apoptosis by reducing mitochondrial Bcl-2 (Wang *et al.* 2005). Wang *et al*. demonstrate that FKBP38 and PSEN1/2 form macromolecular complexes together with the anti-apoptotic Bcl-2. PSEN1/2 thereby promote the degradation of FKBP38 and Bcl-2 and sequester these proteins from the mitochondria to the the ER/Golgi compartments. They suggest that PSEN1/2 increase by this means the susceptibility to apoptosis. Furthermore, FAD-linked PSEN1/2 mutants were shown to enhance the pro-apoptotic activity by causing more efficient reduction in mitochondrial Bcl-2 than wt PSEN1/2. Our previous results on PHD2 regulation by FKBP38 together with this report let us hypothesize that PSEN1/2 might be involved in proteolytic PHD2 regulation and in the regulation of the oxygen sensing pathway in general.

## 3 Original Publication

### Dysregulation of hypoxia-Inducible factor by presenilin/γ-secretase loss-of-function mutations

*Muriel R. Kaufmann,[1] Sandra Barth,[1] Bei Wu,[2] Sascha Egger,[1] Uwe Konietzko,[3] Reiner Kunze,[4] Hugo H. Marti,[4] Meike Hick,[5] Ulrike Müller,[5] Gieri Camenisch,[1] and Roland H. Wenger[1]*

*[1]Institute of Physiology and Zürich Center for Integrative Human Physiology (ZIHP), [2]Center for Neurologic Disease, Harvard Medical School, Boston, MA 02115, [3]Division of Psychiatry Research, University of Zürich, CH-8000 Zürich, Switzerland, [4]Institute of Physiology and Pathophysiology, and [5]Department of Pharmacy and Molecular Biotechnology, University of Heidelberg, D-69120 Heidelberg, Germany*

S. Barth's present address: YES Pharmaceutical Development Services GmbH, D-61381 Friedrichsdorf, Germany

Correspondence should be addressed to Dr. Roland H. Wenger, Institute of Physiology, University of Zürich, Winterthurerstrasse 190, CH-8057 Zürich, Switzerland, Tel.: +41 (0)44 6355065; Fax: +41 (0)44 6356814; E-mail: roland.wenger@access.uzh.ch

Running title: Presenilins regulate PHD/HIF signaling

The authors declare no conflict of interest.

**Abstract**

Presenilin (PSEN) 1 and 2 are the catalytic components of the γ-secretase complex which cleaves a variety of proteins, including the amyloid precursor protein (APP). Proteolysis of APP leads to the formation of the APP intracellular domain (AICD) and amyloid beta that is crucially involved in the pathogenesis of Alzheimer's disease. Prolyl-4-hydroxylase-domain (PHD) proteins regulate the hypoxia-inducible factors (HIFs), the master regulator of the hypoxic response. We previously identified the FK506 binding protein 38 (FKBP38) as a negative regulator of PHD2. Genetic ablation of PSEN1/2 has been shown to increase FKBP38 protein levels. Therefore, we investigated the role of PSEN1/2 in the oxygen sensing pathway using a variety of genetically modified cell lines, mouse tissues and pharmacological inhibitors. Increased FKBP38 protein levels and decreased PHD2 protein levels were found in PSEN1/2-deficient mouse embryonic fibroblasts (MEFs) and in the cortex of forebrain-specific PSEN1/2 conditional double knock-out mice. Hypoxic HIF-1α protein accumulation and transcriptional activity were decreased, despite reduced PHD2 protein levels. Proteolytic γ-secretase function of PSEN1/2 was needed for proper HIF activation. Intriguingly, PSEN1/2 mutations identified in Alzheimer patients differentially affected the hypoxic response, involving the generation of AICD. Taken together, our results suggest a direct role for PSEN in the regulation of the oxygen sensing pathway via the APP/AICD cleavage cascade.

**Introduction**

Hypoxia is a characteristic feature of many neurodegenerative disorders such as familial Alzheimer's disease (FAD) or Parkinson's disease (PD) (reviewed by Ogunshola and Antoniou 2009). The central regulators of the response to low oxygen partial pressures are the hypoxia-inducible factors (HIFs) (Semenza 2007; Wenger *et al.* 2005). Under normoxic conditions, a family of prolyl-4-hydroxylase domain (PHD) proteins hydroxylate the HIF$\alpha$ subunits, tagging them for ubiquitin-mediated proteasomal degradation (Bruick and McKnight 2001; Maxwell *et al.* 1999). When oxygen becomes limiting, the activity of PHDs decreases, resulting in the stabilization and accumulation of HIF$\alpha$ subunits that translocate to the nucleus and heterodimerize with their constitutive counterpart HIF-1$\beta$/aryl hydrocarbon receptor nuclear translocator (ARNT) (Kaelin and Ratcliffe 2008; Schofield and Ratcliffe 2004). The now active HIF complexes regulate genes that are involved in hypoxic adaptation, including glucose metabolism, angiogenesis, cancer progression and neurodegeneration.

Recently, we reported that the peptidyl-prolyl *cis/trans* isomerase FKBP38 specifically interacts with PHD2 and negatively regulates its stability (Barth *et al.* 2009; Barth *et al.* 2007). FKBP38 has been shown previously to interact with presenilin (PSEN) 1 and 2 which antagonizes its antiapoptotic function (Wang *et al.* 2005). PSEN 1 and 2 form the catalytic core of the $\gamma$-secretase complex (De Strooper *et al.* 1998; Wolfe *et al.* 1999). This complex cleaves many type I membrane proteins such as amyloid precursor protein (APP) (De Strooper *et al.* 1998) or Notch (De Strooper *et al.* 1999). PSEN1 and 2 have been discovered through their genetic linkage to early onset FAD (Sherrington *et al.* 1995). Despite thorough research, the underlying pathologic processes causing AD remain elusive. However, tissue hypoxia and hypoperfusion have been linked to the pathological progression of FAD (Ogunshola and Antoniou 2009; Zhang and Le 2010). Vascular ischemia/hypoxia increase APP expression and the production of A$\beta$ (Kalaria *et al.* 1993; Li *et al.* 2009; Webster *et al.* 2002) as well as the amyloidogenic $\beta$-secretase BACE1 (Sun *et al.* 2006; Zhang *et al.* 2007), suggesting that oxygen signaling is also involved in the progression of AD.

The finding that PSEN1/2 deficient mouse embryonic fibroblasts (MEFs) have higher FKBP38 protein levels (Wang *et al.* 2005) led us to hypothesize that presenilins

could be involved in the regulation of the oxygen signaling pathway. To explore a putative role of PSEN1/2 in PHD/HIF regulation, we analyzed wild-type (wt), PSEN1 and PSEN2 single knock-out (ko) as well as PSEN1/2 double-ko MEFs (Herreman *et al.* 1999; Herreman *et al.* 2003). We confirmed that MEF cells lacking PSEN1/2 have increased constitutive FKBP38 protein levels causing decreased PHD2 protein levels. In addition, we found that the lack of PSEN1/2 leads to an attenuated hypoxic response that in turn results in a decreased feedback regulation of PHD2 transcript levels. The observed effects were dependent on the generation of the APP intracellular domain (AICD) but not on the Notch cleavage cascade.

**Materials and Methods**

*Plasmid generation and lentiviral transduction.* psG5-mPSEN1 and pcDNA3.1-mPSEN2 expression plasmids were kind gifts of B. de Strooper (Leuven, Belgium). pcDNA3.1-hPSEN1 was kindly provided by R. Nitsch (Zürich, Switzerland). The pUKBK-citAICD expression vector was described previously (Goodger *et al.* 2009). PSEN and citAICD Entry vectors were generated by cloning PCR fragments into the NcoI/XhoI (for mPSEN1) or EcoRI/XhoI (for mPSEN2) sites of pENTR4 (Invitrogen, Basel, Switzerland). Cloning of pLenti-mPSEN and G4-DBD-PSEN1 constructs was performed using Gateway technology (Invitrogen). The inserts of the Entry vectors were verified by sequencing (Microsynth, Balgach, Switzerland). Viral particles were produced in HEK293T human embryonic kidney cells using the ViraPower lentiviral expression system according to the manufacturer's protocol (Invitrogen).

*Cell culture and transient transfections.* PSEN wt, PSEN1 ko and PSEN1/2 ko, as well as MEFs containing wt human PSEN1 or clinically relevant mutations of PSEN1 (ΔE9, A246E, L166P) or PSEN2 (N141I), were kindly provided by de Strooper (Bentahir *et al.* 2006; Herreman *et al.* 1999; Herreman *et al.* 2003). The HeLa cervix carcinoma cell line HeLa/trTAA/TRE-N1-ICD, capable of doxycycline-induced expression of human Notch1-IC (Lee *et al.* 2009), was a kind gift from C. O. Joe (South Korea). APP ko MEFs and APP/APPLP2 ko MEFs were derived from the corresponding ko mice (von Koch *et al.* 1997; Zheng *et al.* 1995). All cells were grown in high-glucose Dulbecco's modified Eagle's medium (DMEM; Sigma, Buchs, Switzerland) and Notch1-IC

expression was induced by 1 μg/ml doxycycline (Sigma). The HEK293-citAICD cells were described previously (von Rotz *et al.* 2004). Expression of citAICD was induced with 1 μM tebufenozide for 24 hours. For hypoxic exposure, cells were grown in a gas-controlled glove box (Invivo$_2$ 400, Ruskinn Technologies, Leeds, United Kingdom). Transient transfections of MEFs were performed using Lipofectamine2000 (Invitrogen). HeLa cells were transiently transfected using the polyethylenimine (Polysciences, Warrington, PA, USA) method as described previously (Stiehl *et al.* 2006). The specific γ-secretase inhibitor N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT) was purchased from Sigma.

*Immunoblotting*. Immunoblotting was performed as described previously (Martin *et al.* 2005). Primary antibodies used were rabbit anti-APP, C-terminal (Sigma A8717), rabbit anti-human PHD2 (Novus Biologicals, Cambridge, United Kingdom), rabbit anti-mouse PHD2 (Novus Biologicals), rabbit anti-FKBP38 (Edlich *et al.* 2005), rabbit anti-HIF-1α (Novus Biologicals), mouse anti-N-cadherin (BD Biosciences, Heidelberg, Germany), mouse anti-β-actin (Sigma). Horseradish peroxidase coupled secondary anti-mouse and anti-rabbit antibodies were purchased from Pierce (Lausanne, Switzerland). Chemiluminescence detection was performed using Supersignal West Dura (Pierce) and signals were recorded with a charge-coupled device camera (Lightimager LAS-4000mini, Fujifilm, Dielsdorf, Switzerland).

*Reporter genes and mammalian two hybrid assays.* Cloning of the HIF-dependent firefly luciferase reporter gene pH3SVL and the 885 bp mouse *Hif1a* promoter constructs were described previously (Wanner *et al.* 2000; Wenger *et al.* 1997; Wenger *et al.* 1998). Cells were co-transfected with 1 μg pH3SVL and 20 ng pRLSV40 Renilla luciferase reporter vector (Promega, Madison, Wisconsin, USA). Cells were harvested in passive lysis buffer (Promega) and luciferase reporter gene activity was determined using the dual-luciferase reporter system according to the manufacturer's instructions (Promega). Mammalian two hybrid analysis was performed using the mammalian Matchmaker system (Clontech, BD Biosciences) as previously described (Barth *et al.* 2007).

*Animal experimentation.* Male C57BL/6 mice were maintained at the animal facility of the University of Heidelberg, Germany. All animal procedures were approved

by the animal welfare committee (Regierungspräsidium Karlsruhe, Germany). Hypoxia was induced in adult mice by substituting nitrogen for oxygen using a Digamix 5SA 18/3A pump (Woesthoff, Bochum, Germany). Mice breathed 8% $O_2$ for 2 to 108 hours or were kept at room air. After hypoxic exposure, organs were removed and snap frozen in liquid nitrogen. The generation of fPSEN1/fPSEN1;αCaMKII-Cre;PSEN2ko mice was described previously (Saura *et al.* 2004).

*RNA extraction and quantification.* Total RNA from cells or from the cortex of CaMKIIcre PSEN1/2 cdko mice was extracted as described previously (Wollenick *et al.* 2011). First-strand cDNA synthesis was performed with 1.5 μg RNA using affinity script reverse transcriptase (RT) enzyme (Stratagene, Amsterdam, Netherlands). mRNA expression levels were quantified by real-time quantitative (q) PCR using a SybrGreen qPCR reagent kit (Sigma) and a MX3000P cycler (Stratagene). Initial template concentrations of each sample were calculated by comparison with serial dilutions of a calibrated standard. To control for equal input levels, ribosomal protein S12 or L28 mRNA was determined and data were expressed as relative ratios.

*Data analysis.* Unless otherwise indicated, results are shown as mean values ± standard error of the mean (SEM) of at least three independent experiments. Statistical analysis was performed applying two-tailed Student's *t*-test using GraphPad Prism version 4.0 (GraphPad Software, La Jolla, California, USA).

## Results

### PSEN 1 and 2 regulate constitutive FKBP38 and PHD2 levels and are ubiquitously expressed

To explore the function of PSEN1/2 in the hypoxia signaling pathway, PSEN1 and PSEN2 single as well as PSEN1/2 double ko MEFs (MEF PSEN1 ko, and MEF PSEN1/2 ko, respectively) were used (Herreman *et al.* 1999; Herreman *et al.* 2003). As determined by immunoblotting, constitutive FKBP38 protein levels were increased whereas PHD2 protein levels were strongly downregulated in PSEN1/2 ko MEFs (Fig. 1A, left panel). Quantification of relative band intensities of three independent experiments revealed 1.7-fold FKBP38 upregulation and 5-fold PHD2 downregulation (Fig. 1A, right panel). This finding is consistent with a previous report that the lack of

PSEN1/2 leads to increased FKBP38 protein abundance as well as with our published results on the negative regulation of PHD2 protein stability by FKBP38 (Barth *et al.* 2007; Wang *et al.* 2005).

Since FKBP38 was clearly less increased than PHD2 was decreased in PSEN1/2 ko MEFs, it is likely that at least one additional mechanism is involved in the regulation of PHD2 in cells lacking PSEN1/2. RT-qPCR analysis demonstrated that PHD2 mRNA levels were also significantly lower in PSEN1/2 ko MEFs whereas there was no difference in FKBP38 mRNA levels (Fig. 1B). We further analyzed hypoxic PHD2 protein induction in PSEN1/2 wt and ko MEFs. Both, normoxic and hypoxic PHD2 protein levels were substantially lower in the absence of PSEN1/2 at each timepoint tested (Fig. 1C). FKBP38 protein levels did not change in hypoxia, like reported previously (Barth *et al.* 2007). Relative hypoxic PHD2 induction, however, remained unchanged when compared to normoxic PHD2 protein levels in each cell line (Fig. 1D).

*In vivo*, we found ubiquitous PSEN1/2 expression in adult mouse tissues with most abundant PSEN1 mRNA levels in the testis and the brain, and with high PSEN2 mRNA levels in the liver and the brain (Fig. 1E). While exposure to inspiratory hypoxia (8% $O_2$) for 12 or 108 hours induced mRNA levels of the HIF-target gene EPO in the brain by 25-fold and 26-fold, respectively, and in the kidney by 168-fold and 53-fold, respectively (data not shown), no hypoxic induction of PSEN1 or PSEN2 itself could be detected, neither in these mouse tissues (Fig. 1*E*), nor in various human cell lines (data not shown).

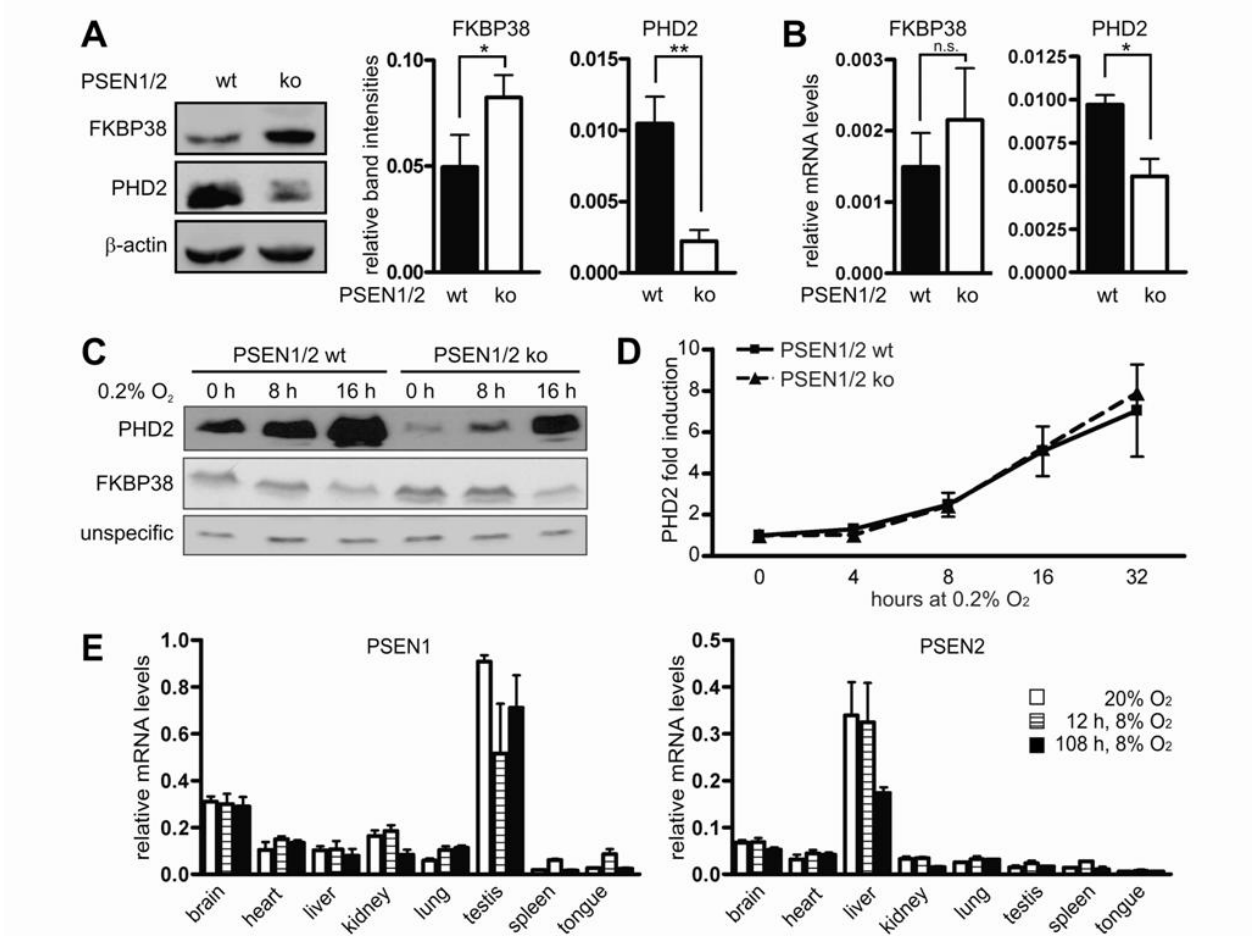


***Fig.1. Regulation of FKBP38 and PHD2 by PSEN1 and PSEN2 in normoxia and hypoxia.*** *(**A**) Total cell extracts from PSEN1/2 wt and ko MEFs were analyzed for PHD2, FKBP38 and β-actin protein levels by immunoblotting (left panel). Relative band intensities of three independent experiments were quantified by densitometry (right panel). Data are shown as mean values ± SD; *p < 0.05, **p < 0.005, t-test. (**B**) Total RNA was extracted from PSEN1/2 wt and ko MEFs. Transcript levels of FKBP38 and PHD2 were quantified by RT-qPCR and normalized to ribosomal protein S12 mRNA levels. Data are presented as mean values ± SD; n.s. not significant, *p < 0.05, t-test. (**C**) PSEN1/2 wt and ko MEFs were cultured in normoxia or hypoxia for the time indicated and PHD2 and FKBP38 protein levels were analyzed by immunoblotting. (**D**) Relative band intensities of three independent experiments were quantified by densitometry. The 0 hours timepoint of each cell line was defined as 1. (**E**) Total RNA was derived from organs of mice that were kept at 20% or 8% oxygen for the time indicated. PSEN1 and PSEN2 transcript levels were quantified by RT-qPCR and normalized to the ribosomal protein S12 mRNA levels. Data are shown as mean values ± SEM of three independent RNA extractions from different mice.*

## PSEN deficient cells show an attenuated hypoxic response

To investigate whether the constitutive decrease in PHD2 levels in PSEN1/2 deficient cells affects the hypoxic response functionally, wt, single ko, double ko, and lentivirally reconstituted MEFs were transiently co-transfected with a hypoxia response element (HRE)-driven firefly luciferase reporter gene together with a constitutive Renilla

luciferase control vector. After transfection, cells were split and exposed to normoxia or hypoxia for 16 hours. Unexpectedly, despite decreased PHD2 levels both hypoxic HIF-1$\alpha$ protein levels and HIF-dependent reporter gene expression were decreased in PSEN1/2 ko MEFs (Fig. 2A). Reconstitution by lentiviral re-expression of wt PSEN1 (C1) or PSEN2 (C2) almost completely rescued the hypoxic HIF-1$\alpha$ protein levels and reporter gene induction, excluding clonal artifacts and demonstrating that both PSEN1 and PSEN2 are independently involved in HIF regulation. Hypoxic induction of mRNA levels derived from the well-established HIF-target genes carbonic anhydrase IX (CaIX), PHD2, N-myc downstream regulated gene (NDRG1) and BCL2/adenovirus E1B interacting protein 3 (BNIP3) was blunted in PSEN1/2 ko MEFs (Fig. 2B), confirming a functional decrease of endogenous HIF activity in the absence of PSEN1/2.

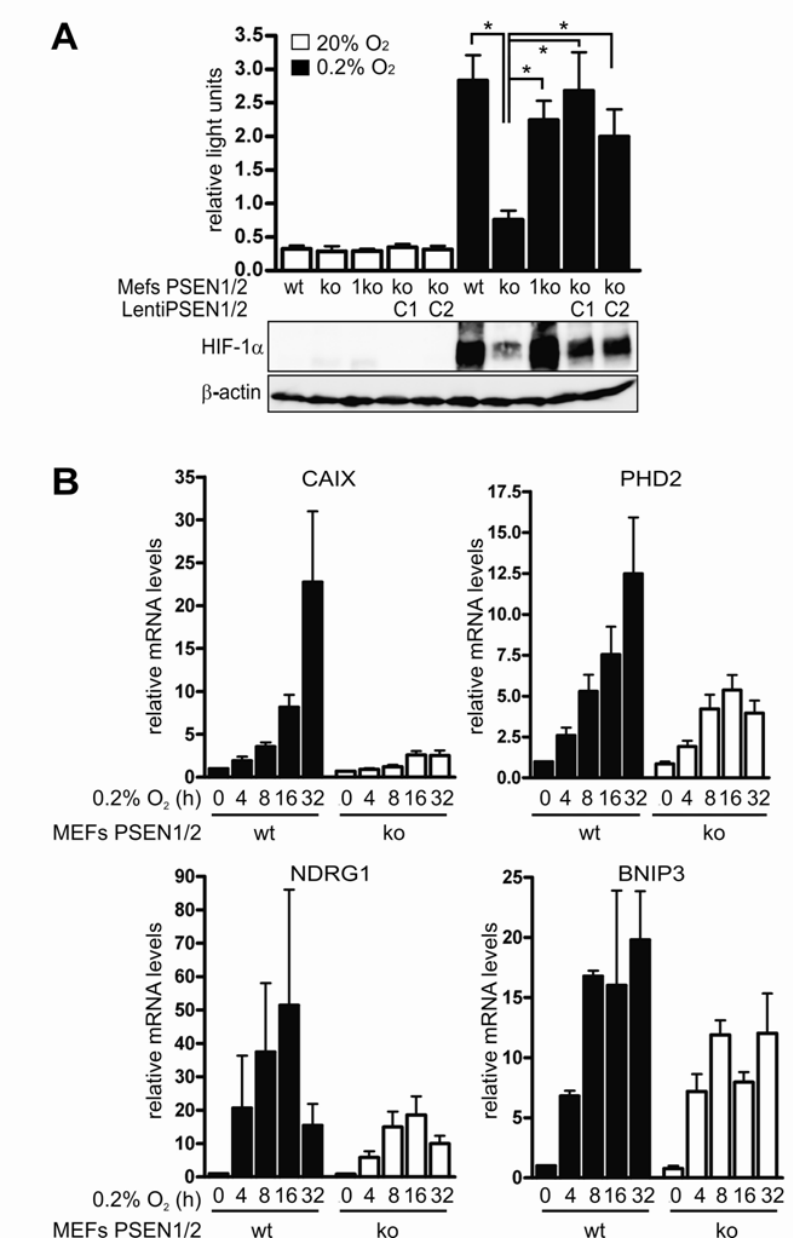


***Fig.2. HIF transcriptional response to hypoxia in PSEN1/2 deficient cells.*** *(**A**) wt, PSEN1/2 ko (ko), PSEN1 ko (1ko) and two reconstituted clones of PSEN1/2 ko (C1 or C2) MEFs were transiently transfected with the HIF-dependent reporter pH3SVL and pRL-SV40 constructs and cultured in 20% or 0.2% $O_2$ for 16 hours before relative luciferase activities were determined. The results are shown as mean values ± SEM of three independent experiments performed in triplicates (top panel); *p < 0.05, t-test. HIF-1α and β-actin protein levels were determined by immunoblotting (bottom panel). (**B**) PSEN1/2 wt and ko MEFs were cultured in 20% or 0.2% $O_2$ for 4, 8, 16 and 32 hours and total RNA was extracted. CAIX, PHD2, NDRG1 and BNIP3 transcript levels were quantified by RT-qPCR and normalized to the expression of ribosomal protein S12 mRNA. Data are shown as mean values ± SEM of five independent experiments.*

**PSEN1 is required for normal *Hif1a gene* expression**

To further analyze the PSEN1/2 dependent regulation of the hypoxia pathway, HIF-1α protein stability was examined. Since in normoxia HIF-1α is virtually undetectable due to rapid hydroxylation-dependent proteasomal degradation, HIF-1α protein decay was estimated under hypoxic conditions only. After 16 hours of hypoxia, the translation inhibitor cycloheximide (CHX) was added to PSEN1/2 wt and ko MEFs. HIF-1α protein levels were analyzed by immunoblotting after 0, 0.5, 1, 2 and 4 hours of ongoing hypoxia and cycloheximide treatment (Fig. 3A). Quantification of the protein degradation rate of three independent experiments revealed that the hypoxic half-life of HIF-1α protein was around 2.5 hours and did not differ between the two cell lines (Fig. 3B).

Since HIF-1α protein stability was not altered in a PSEN ko MEFs, HIF-1α mRNA levels were determined by RT-qPCR. Transcript levels in PSEN1/2 ko MEFs were decreased by almost 50% compared to the wt control (Fig. 3C). To determine HIF-1α mRNA stability in PSEN1/2 wt and ko MEFs, cells were treated with actinomycin D for up to 24 hours and transcript levels quantified by RT-qPCR. Neither HIF-1α, PHD2 nor VEGFA mRNA stability was found to be altered in PSEN1/2 ko MEFs (Fig. 3D), suggesting that HIF-1α mRNA is transcriptionally regulated and not via degradation.

Because *Hif1a* promoter activity likely is responsible for the difference in HIF-1α mRNA levels, a previously published 885 bp fragment upstream of the transcriptional start site of the mouse *Hif1a* gene, cloned into a promoterless firefly luciferase reporter gene vector (Wenger *et al.* 1998), was used to determine HIF-1α transcription rates. A co-transfected pSV40-Renilla vector served as an internal control to correct for differences in transfection efficiency and extract preparation. Luciferase expression was determined 48 hours after transfection and values were normalized to the activity of the promoterless vector pGL3basic in wt MEFs. As shown in Fig. 3E, *Hif1a* promoter activity was significantly lower in PSEN1/2 ko than in wt MEFs. *Hif1a* promoter activity was rescued to almost wt levels in two different clones of ko MEFs that were lentivirally reconstituted with PSEN1/2.

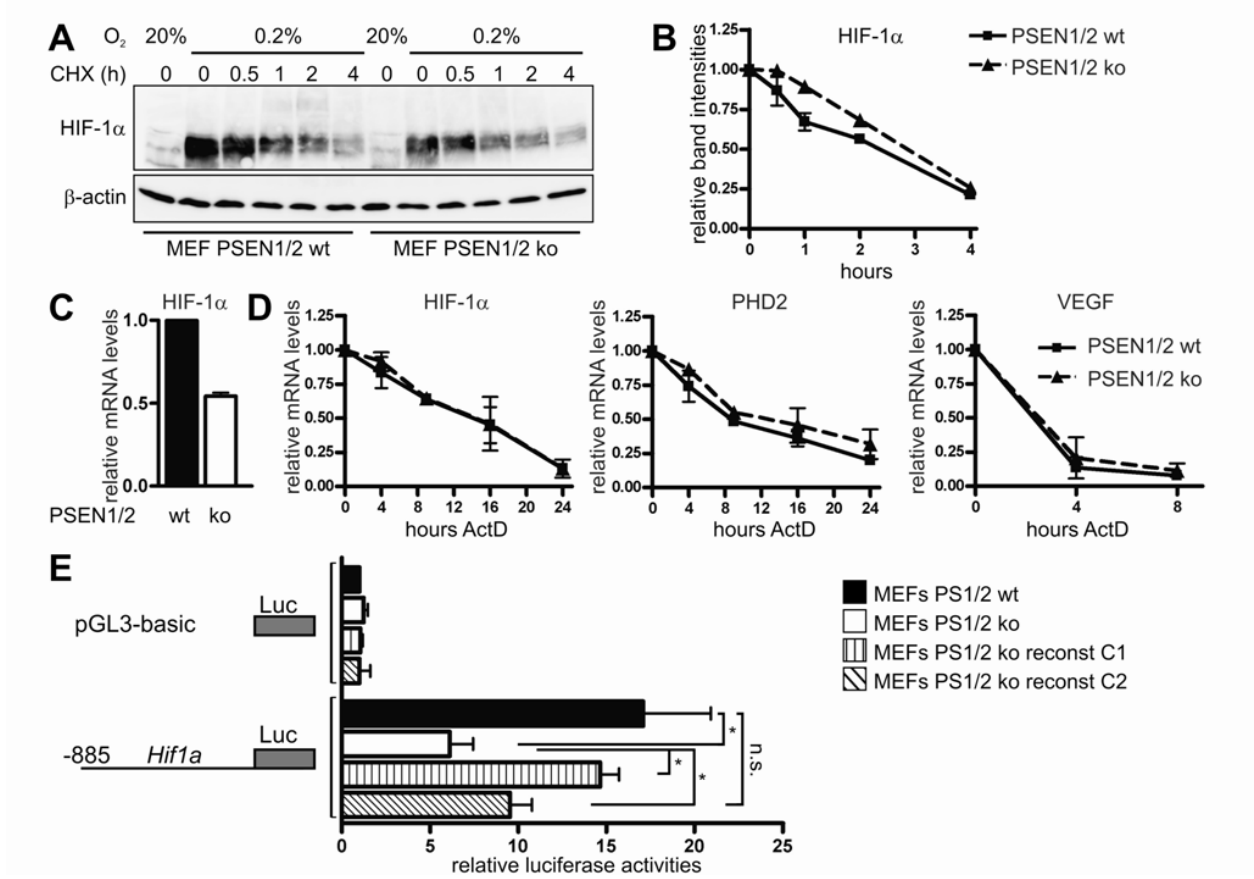


***Fig.3. HIF-1α regulation in PSEN1/2 deficient cells****. (**A**) PSEN1/2 wt and ko MEFs were cultured in 20% or 0.2% $O_2$ for 16 hours before treatment with 100 μM CHX. Total cell extracts were prepared after 0.5, 1, 2 and 4 hours of treatment. HIF-1α, PHD2 and β-actin protein levels were analyzed by immunoblotting. (**B**) Relative band intensities of three independent experiments were quantified relative to the β-actin levels and normalized to the 0 hours hypoxia time points. Mean values ± SEM of three independent experiments are shown. n.s. = not significant, t-test. (**C**) Quantification of HIF-α mRNA levels in PSEN1/2 wt and ko MEFs by RT-qPCR. Transcript levels were normalized to the mRNA levels of ribosomal protein S12 and the wt level was defined as 1. (**D**) PSEN1/2 wt and ko MEFs were incubated at 0.2% $O_2$ for 16 hours before 5 μg/ml actinomycin D was added to the cells. Total RNA was extracted after 0, 4, 8, 12 and 16 hours of actinomycin D treatment and HIF-1α, PHD2 and VEGFA transcript levels were quantified by RT-qPCR. mRNA levels were normalized to ribosomal protein S12 mRNA and the 0 hours time point was defined as 1. The results are shown as mean values ± SEM of three independent experiments. (**E**) PSEN1/2 wt, ko and two PSEN1/2 reconstituted clones were transiently co-transfected with the Hif1a promoter-driven pGL3-885Hif1a or the promoterless pGL3-basic plasmid together with the pSV40-RL control vector. Data were normalized to the Renilla luciferase activities and are shown as mean values ± SEM of three independent experiments performed in triplicates; *p < 0.05, t-test.*

**γ-Secretase enzymatic activity is required for PSEN dependent regulation of HIF-1α but not of FKBP38/PHD2**

Most of the known functions of PSEN1/2 require γ-secretase proteolytic activity (Wakabayashi and De Strooper 2008). To determine whether γ-secretase activity is also required for the regulation of the hypoxia pathway, PSEN1/2 wt and ko MEFs were cultured under normoxic or hypoxic conditions for 12 hours in the presence of either DMSO alone (control) or 2 μM or 4 μM DAPT, a specific γ-secretase inhibitor. As shown in Fig. 4A, inhibition of γ-secretase activity reduced HIF-1α protein levels but did not have any effect on FKBP38 or PHD2 protein levels. The accumulation of the C-terminal fragment of N-cadherin served as a control for DAPT function. This finding further supports that the regulation of the hypoxia pathway takes place on more than one level, comprising γ-secretase dependent and γ-secretase independent mechanisms.

Notch is one of the many γ-secretase substrates and has been described to potentiate the hypoxic response in certain cell lines on various levels, involving the direct interaction of HIF-1α with Notch intracellular domain (NICD) and the recruitment of HIF-1α to NICD responsive promoters (Gustafsson *et al.* 2005). To analyze if PSENs act on the hypoxia pathway via Notch signaling, HeLa/trTAA/TRE-N1-IC cells were used, allowing induced expression of the human Notch1-IC by doxycycline (Lee *et al.* 2009). Protein levels of doxycycline or vehicle treated HeLa cells were analyzed after 16 hours exposure to 21% or 0.2% $O_2$. As shown in Fig. 4B, hypoxic accumulation of HIF-1α and PHD2 were not affected by NICD induction. Consistently, while PHD2, CAIX and GLUT1 mRNA levels were induced 2.5 to 100-fold after 16 hours of hypoxic exposure, they remained unchanged by NICD induction (Fig. 4C). Increased SNAIL mRNA levels confirmed the activation of the Notch pathway (Fig. 4C). These findings suggest that PSEN dependent HIF regulation in our models is not due to alterations in the Notch signaling pathway.

Gain-of-function as well as (partial) loss-of-function mutations in PSEN1/2 are strongly associated with FAD. Therefore, we sought to analyze the effects of several clinically relevant PSEN mutations on the hypoxia pathway by using PSEN1/2 wt and ko MEFs, or ko MEFs stably reconstituted with human PSEN mutants commonly found in

FAD patients (PSEN1-ΔE9, PSEN1-A246E, PSEN1-L166P, PSEN1-G384A and PSEN2-NI41I) (Bentahir *et al.* 2006). These cells were transiently transfected with the HIF-dependent reporter gene pH3SVL and luciferase activity was measured after 16 hours of exposure to either 20% or 0.2% $O_2$. While re-introduction of PSEN1-A246E, PSEN1-G384A and PSEN2-N141I rescued HIF activity in hypoxic PSEN1/2 ko MEFs, PSEN1-ΔE9 and PSEN1-L166P failed to do so (Fig. 4D). Of note, whereas the former three mutations are still able to cleave some substrates (e.g. APP) but not others (e.g. Notch), γ-secretase function of the latter two mutations has been shown to be severely impaired and to abolish canonical APP cleavage as well as NICD formation (Bentahir *et al.* 2006), confirming the results obtained with the DAPT γ-secretase inhibitor.

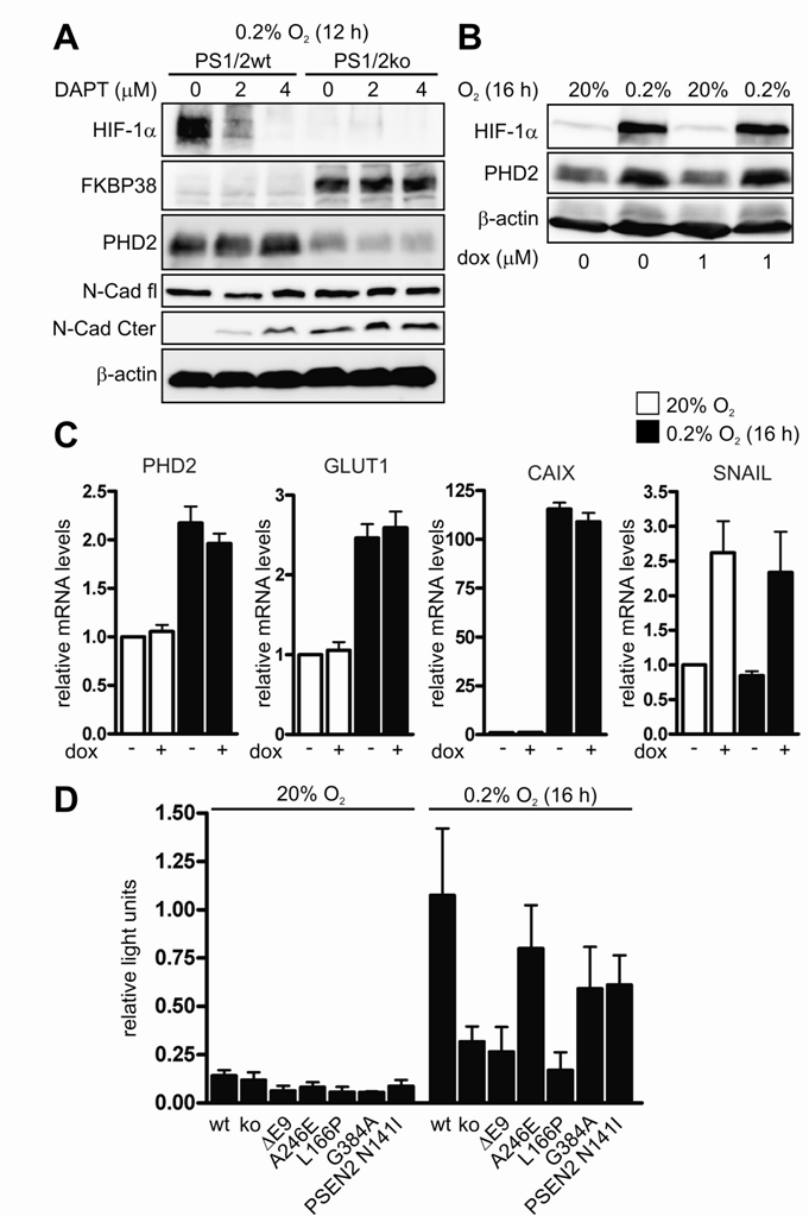


***Fig.4. Requirement of γ-secretase enzymatic activity for presenilin dependent regulation of HIF-1α, FKBP38 and PHD2****. (**A**) PSEN1/2 wt and ko MEFs were pretreated with 0, 2 or 4 μM of the γ-secretase inhibitor DAPT before culturing at 0.2% $O_2$ for 12 hours. HIF-1α, FKBP38, PHD2, N-cadherin and β-actin protein levels were determined by immunoblotting. (**B**, **C**) HeLa/trTAA/TRE-N1-ICD cells were cultured for 24 hours in the presence or absence of 1 μM doxycycline before exposure to 20% or 0.2% $O_2$ for 16 hours. Thereafter, HIF-1α, PHD2 and β-actin protein levels were determined by immunoblotting (**B**) and PHD2, GLUT1, CAIX and Snail mRNA levels were quantified by RT-qPCR (**C**). Transcript levels were normalized to the mRNA levels of ribosomal protein L28. The untreated normoxic control was defined as 1 and data are shown as mean values ± SEM of three independent experiments. (**D**) PSEN1/2 wt, ko and ko MEFs stably expressing PSEN1 or PSEN2 FAD mutations (ΔE9, A246E, L166P, G384A, N141I) were transiently cotransfected with the HRE-driven luciferase reporter plasmid (pH3SVL) together with the pSV40-RL control vector. Cells were cultured for 16 hours at 20% or 0.2% $O_2$ before luciferase activity was determined. The results are shown as mean values ± SEM of three independent experiments performed in triplicates.*

**Regulation of HIF-1α protein and mRNA levels via APP/AICD**

The finding that MEFs bearing PSEN mutations that are unable to generate the NICD but still cleave the APP show a functional hypoxic response led us to hypothesize that AICD might be crucial for HIF regulation. Therefore, HIF-1α protein levels were determined in HEK293-citAICD, a human embryonic kidney cell line that allows for tebufenozide induction of AICD expression (von Rotz *et al.* 2004). Following tebufenozide treatment, HIF-1α but not PHD2 protein levels were increased (Fig. 5A). since normoxic HIF-1α protein is rapidly degraded and therefore not detectable in the left panel of Fig. 5A, camera exposure time of the immunoblot of HEK293-citAICD cells cultured in 20% $O_2$ was increased. As shown in Fig. 5A right panel, normoxic HIF-1α protein levels were induced after AICD overexpression. On the mRNA level, HIF-1α was increased by 70% in normoxia and to a lesser extent in hypoxia after AICD induction (Fig. 5B). Hypoxic CAIX levels were clearly over-induced after AICD induction whereas the effect on PHD2 mRNA levels was rather mild. AICD overexpression was verified by immunoblotting (Fig. 5A) as well as RT-qPCR (Fig. 5B).

To further explore the involvement of APP/AICD in the hypoxic response, we analyzed HIF-1α and PHD2 protein levels in APP wt, APP ko and APP/APP like protein 2 (APLP2) double ko MEFs by immunoblot analysis. APLP2 has been shown to be able to partially take over the function of APP (Heber *et al.* 2000; Yang *et al.* 2005). Hypoxic protein accumulation was blunted in the absence of APP and even further decreased in APP/APLP2 ko MEFs (Fig. 5C). Normoxic and hypoxic transcript levels of HIF-1α were lower in APP/APLP2 MEFs and hypoxic induction of CAIX was blunted in APP ko and APP/APLP2 ko MEFS whereas PHD2 was not affected (Fig. 5D). Finally, we investigated if AICD overexpression is sufficient to rescue the blunted hypoxic response following γ-secretase inhibition. Therefore, AICD overexpression in Hek293-citAICD cells was induced for 16 hours before γ-secretase activity was blocked by DAPT treatment for 8 hours and the cells incubated at 20% or 0.2% $O_2$. While AICD overexpression resulted in increased hypoxic HIF-1α protein levels, γ-secretase inhibition reduced HIF-1α but not PHD2 protein levels in the presence and absence of AICD (Figs. 5E and F). These findings indicate that the APP/AICD cleavage cascade is

involved in PHD/HIF signaling but is likely not the only γ-secretase substrate involved in these effects.

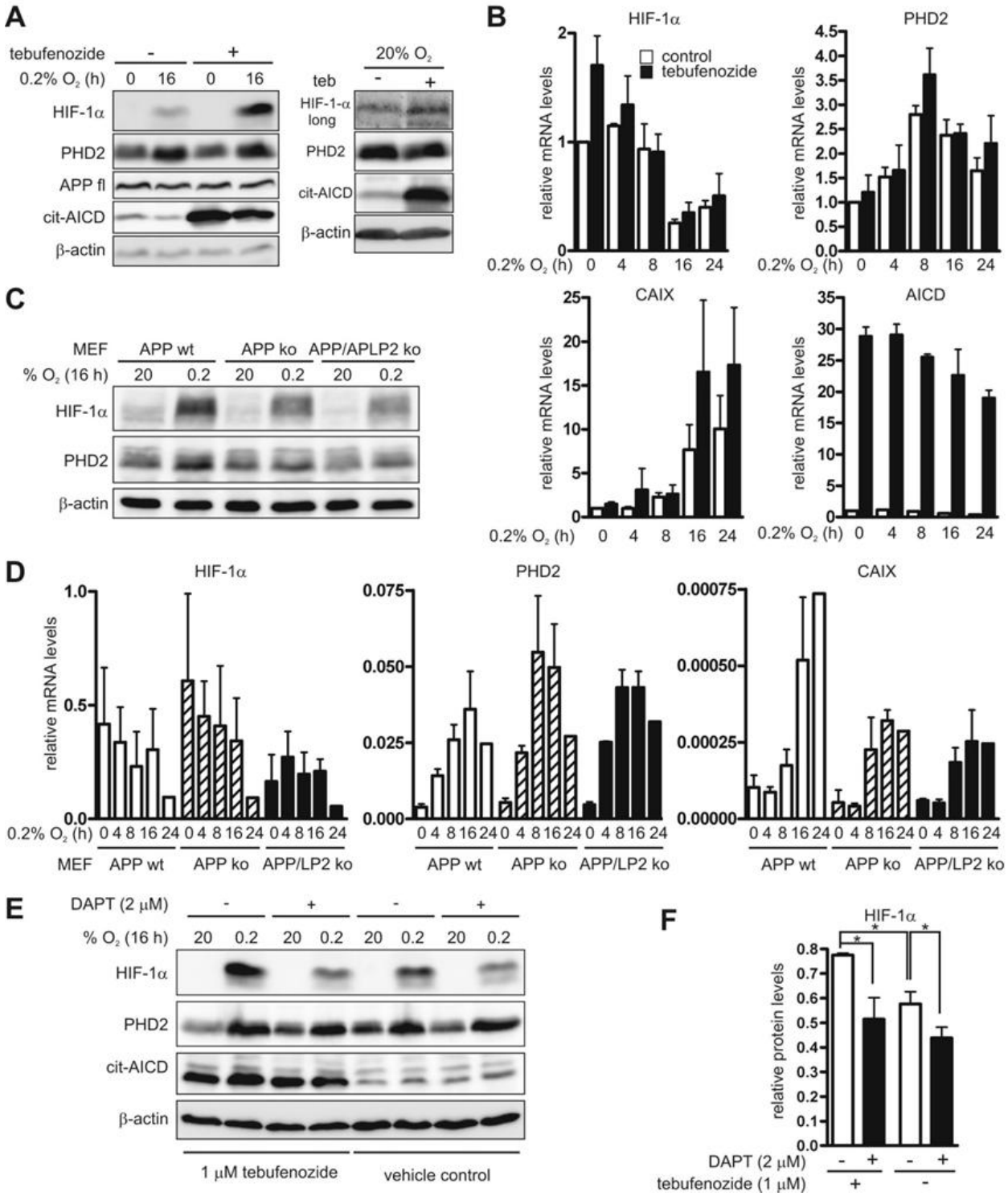


***Fig.5. Regulation of HIF by the APP/AICD cleavage cascade.*** *(**A**) Hek293-citAICD cells were pretreated with 1 μM tebufenozide for 24 hours before culturing at 20% or 0.2% $O_2$ for 16 hours and determination of HIF-1α, PHD2, APP and β-actin by immunoblotting (left panel). Long immunoblot*

*exposure for normoxic HIF-1α in Hek293-citAICD cells with or without tebufenozide treatment (right panel). (**B**) Hek293-citAICD cells were cultured for 24 hours in the presence or absence of 1 μM tebufenozide and exposed to 20% or 0.2% $O_2$ for the time indicated, before mRNA levels of HIF-1α, PHD2, CAIX and AICD were quantified by RT-qPCR. The transcript levels were normalized to ribosomal protein L28 mRNA levels and the zero hour timepoint of the control cells was defined as 1. (**C**) APP wt, APP ko and APP/APPLP2 ko MEFs were cultured in 20% or 0.2% $O_2$ for 16 hours and HIF-1α, PHD2 and β-actin protein levels were determined by immunoblotting. (**D**) APP wt, APP ko and APP/APLP2 ko MEFs were exposed to 0, 4, 8, 16 or 24 hours of 0.2% $O_2$ and mRNA levels of HIF-1α, PHD2 and CAIX were quantified by RT-qPCR. Transcript levels were normalized to ribosomal protein S12 mRNA levels. (**E**, **F**) Hek293-citAICD cells were grown in the presence or absence of 1 μM tebufenozide for 16 hours before 8 hours pretreatment with DMSO or DAPT and subsequent incubation at 20% or 0.2% $O_2$ for 16 hours. HIF-1α, PHD2 and AICD were determined by immunoblotting (**E**) and HIF-1α band intensities were quantified and normalized to β-actin. (**F**). All data are shown as mean values ± SEM of three independent experiments; *p < 0.05, t-test.*

## Decreased HIF-1α expression in the cortex of forebrain-specific PSEN1/2 conditional double knock-out mice

To confirm the relevance of our findings *in vivo*, we analyzed the cortex region of forebrain-specific fPSEN1/fPSEN1;αCaMKII-Cre;PSEN2ko PSEN1/2 conditional double knock-out (PSEN1/2 cdko) mice (Saura *et al*., 2004). HIF-1α, HIF-2α, PHD2 and VEGFA mRNA levels were found to be significantly downregulated in the cortex of these mice, whereas there was no difference in FKBP38 and the HIF-target gene NDRG1 mRNA levels (Fig. 6A). In line with the mRNA levels, PHD2 protein levels were decreased by approx. 65% whereas FKBP38 protein remained unaffected in the cortex of PSEN1/2 cdko mice (Figs. 6B and C). Furthermore, we investigated BACE1 expression that was shown to be induced in hypoxia by HIF-1α (Zhang *et al*., 2007). In line with the decreased HIF-1α mRNA levels, BACE1 expression was lower in the brains of PSEN1/2 cdko mice (Fig. 6A), suggesting that reduced Aβ production in PSEN1/2 cdko mice could be mediated via HIF-1α and BACE1.

In summary, our findings demonstrate that PSEN1/2 are involved in the regulation of the oxygen sensing pathway in cells of various origins *in vitro* as well as in cortical neurons *in vivo*.

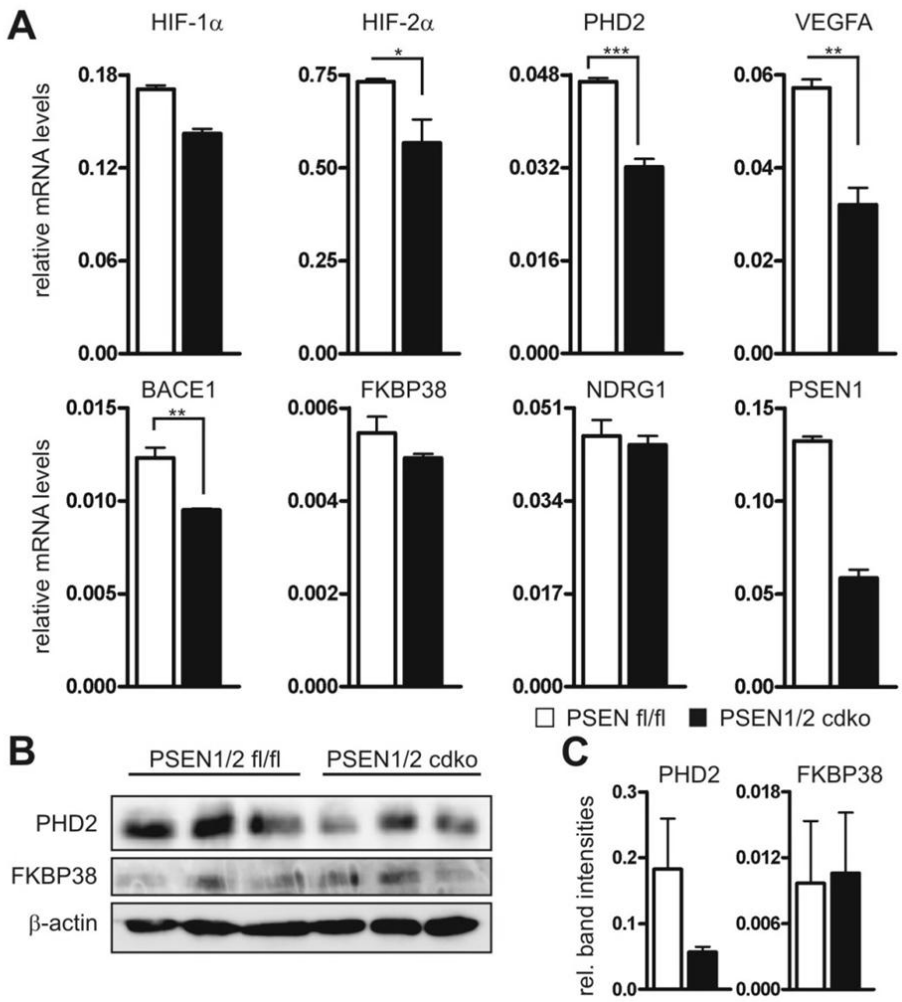


***Fig.6. PSEN1/2 regulate PHD2/HIFα in the brain.*** ***(A)*** *Cortical mRNA of control C57BL6/129 and fPSEN1/fPSEN1;αCaMKII-Cre;PSEN2ko mice was quantified by RT-qPCR and normalized to the transcript levels of the ribosomal protein S12. PHD2, FKBP38 and β-actin protein levels were determined by immunoblotting* ***(B)*** *and PHD2 and FKBP38 band intensities were quantified and normalized to β-actin* ***(C)****. All data are shown as mean values ± SEM of n = 3 animals per group; *p < 0.05, **p < 0.01, ***p < 0.001, t-test.*

## Discussion

Many neurological diseases such as AD and PD are characterized by tissue hypoxia, but whether hypoxia is a cause or a consequence of the disease remains unclear (Peers *et al.* 2009). In this report, we show that PSEN1 and PSEN2 modulate the functional response to hypoxia on more than one level (schematically summarized in Fig. 7).

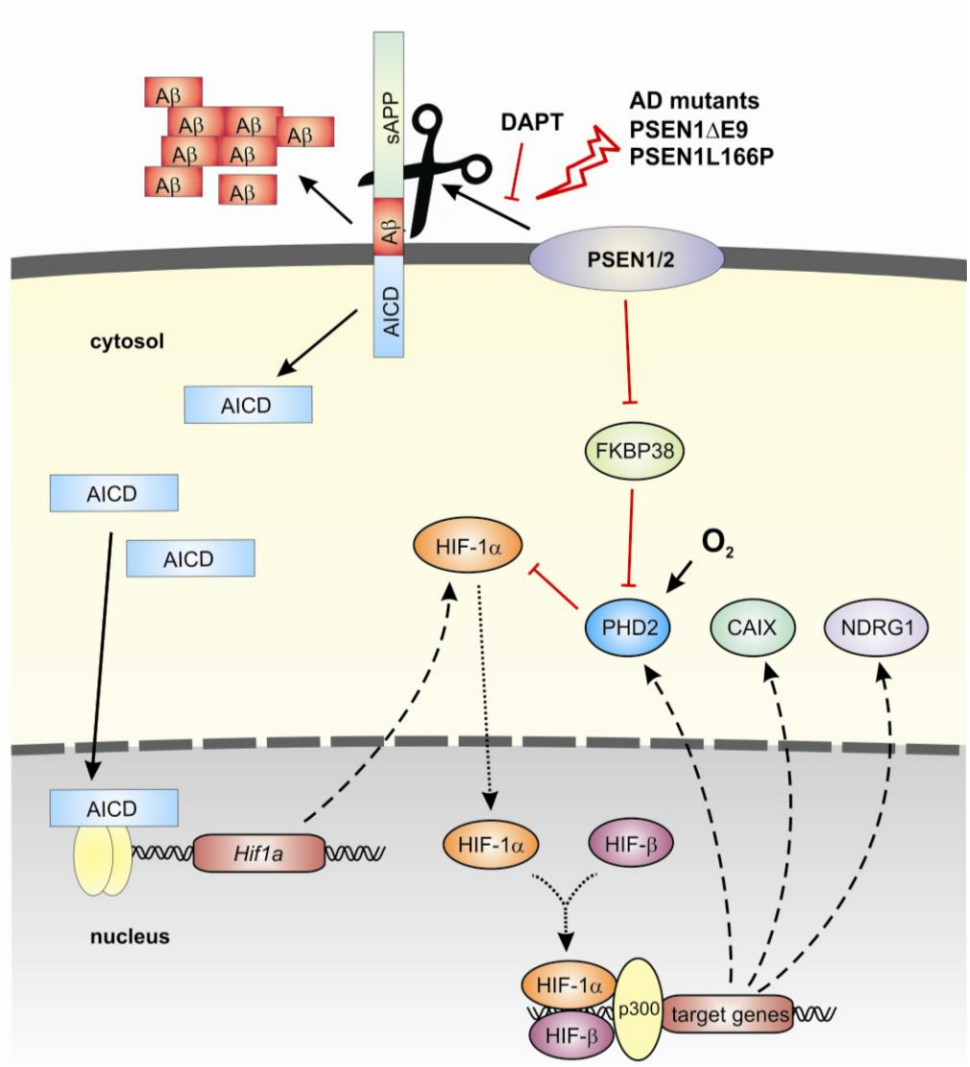


***Fig.7. Scheme of the mechanisms involved in the regulation of HIF by PSENs***. *PSEN1/2 γ-secretase-mediated cleavage of the APP generates Aβ involved in AD as well as the AICD that induces Hif1a gene expression and HIF-1α protein stability but does not regulate FKBP38/PHD2. On the other hand, PSEN1/2 increases PHD2 activity by inhibiting FKBP38 in a γ-secretase-independent manner. These two mechanisms overlap with hypoxic induction of HIF-1α protein stability and finally converge in the downregulation of HIF-dependent target gene expression following deletion or functional mutation of PSEN1/2.*

MEFs lacking PSEN1/2 showed increased constitutive FKBP38 and concomitantly decreased PHD2 protein levels. These findings are in line with a previous report, showing that genetic ablation of PSEN1/2 increases FKBP38 protein levels (Wang *et al.* 2005), and with our previous results showing that FKBP38 negatively regulates PHD2 protein levels (Barth *et al.* 2009). Neither PSEN1 nor PSEN2 themselves were regulated by hypoxia in any of the mouse tissues or cell lines analyzed, even though some reports showed an induction of PSEN1 after hypoxic exposure of glial cells (Bazan and Lukiw 2002; Cui *et al.* 2004). However, FKBP38 protein levels were only slightly upregulated, suggesting that additional mechanisms contribute to the decreased PHD2 levels. Consistently, not only PHD2 protein but also mRNA levels were substantially downregulated in PSEN1/2 deficient MEFs.

Because we and others previously showed that even slight changes in PHD2 protein levels affect HIF-1$\alpha$ protein stability (Ginouvès *et al.* 2008; Henze *et al.* 2010; Stiehl *et al.* 2006), the clearly reduced PHD2 levels in PSEN1/2 deficient MEFs led us to expect that HIF-1$\alpha$ protein and HIF target gene expression will be induced in these cells. However, quite surprisingly PSEN1/2 deficient MEFs also showed a decreased HIF response, despite the strongly decreased PHD2. Since PHD2 is a direct target of HIF-1$\alpha$, these findings might provide the additional mechanism involved in PHD2 downregulation, but they cannot explain the HIF-1$\alpha$ downregulation. PHD3 protein levels were not affected by the loss of PSEN1/2 while PHD1 expression was almost undetectable in PSEN1/2 wt and ko MEFs (data not shown).

Interestingly, the lack of PSEN1/2 lowered both *Hif1a* promoter activity and HIF-1$\alpha$ mRNA levels in MEFs. In line with the *in vitro* data, HIF-1$\alpha$, HIF-2$\alpha$, PHD2 and VEGFA RNA and PHD2 protein levels were decreased in the cortex of forebrain-specific PSEN1/2 cdko mice. These *in vitro* and *in vivo* results confirm previous descriptive *in vivo* gene array data demonstrating that HIF-1$\alpha$ mRNA levels are decreased in PSEN1 hypomorphic mice (Liauw *et al.* 2002; Rozmahel *et al.* 2002). However, it is currently unclear whether the 50% decrease in HIF-1$\alpha$ mRNA levels alone could explain the more pronounced (80%) decrease in hypoxic HIF-1$\alpha$ protein. During the progression of our study, Gasperi *et al.* showed that PSEN1 ko MEFs display impaired induction of HIF-1$\alpha$ following stimulation with hypoxia mimetics, insulin and calcium chelators (De

Gasperi *et al.* 2010). These authors attributed their findings to a shorter half live of HIF-1α in PSEN1-deficient MEFs. However, using PSEN1/2-deficient MEFs, we could not find any decrease in HIF-1α protein stability. Two main differences between our experiments and those performed by Gasperi *et al.* might explain this discrepancy: first, we conducted our analysis in double-ko MEFs; and second we measured HIF-1α decay following a controlled hypoxic accumulation since HIF-1α is normally not detectable under normoxic conditions.

Together with PSEN enhancer (Pen-2), nicastrin, and anterior pharynx 1 (Aph-1), PSENs form the core of the γ-secretase complex that cleaves a variety of type I transmembrane proteins (De Strooper *et al.* 1999; Parks and Curtis 2007). Several studies implied that PSEN also have γ-secretase independent functions (reviewed by Wakabayashi and De Strooper 2008). Accordingly, we showed that whereas the γ-secretase function of PSEN1/2 is essential for hypoxic HIF-1α induction it does not affect the protein levels of FKBP38 and PHD2, further underlining that at least two independent mechanisms are involved in PSEN-dependent HIF regulation.

NICD and AICD are two major γ-secretase dependent intracellular cleavage products (derived from the Notch and APP precursors, respectively) and NICD has been shown previously to potentiate the HIF response in neuronal and myogenic cells (Gustafsson *et al.* 2005). However, in the cell models used in this study AICD but not NICD regulated HIF-1α and HIF target gene expression. The function of AICD remains incompletely understood but it has been shown to be present in nuclear complexes involved in nuclear signaling (Cao and Sudhof 2001; von Rotz *et al.* 2004). Of note, several genes regulated by AICD have been identified, including KAI1 (Baek *et al.* 2002), glycogen synthase 3β (Kim *et al.* 2003), APP and BACE (von Rotz *et al.* 2004). Our results suggest that HIF-1α might represent a novel AICD target gene but further mechanistic experiments need to be performed to corroborate this hypothesis. Since γ-secretase inhibition decreased hypoxic accumulation even in the presence of overexpressed AICD, other γ-secretase substrates are likely to be involved in HIF regulation. A recent report by (Le Moan *et al.* 2011) showed that γ-secretase activity is increased in hypoxia by an unknown mechanism, leading to increased HIF-1α stability.

Even though PSEN1/2 was not investigated, the authors found that genetic ablation of the γ-secretase substrate p75 neurotrophin receptor reduced hypoxic HIF-1α stabilization. Their findings were explained through decreased Siah2 abundance after p75 abrogation. Siah2 has been proposed previously to negatively regulate PHD1 and PHD3, and decreased Siah2 hence would lead to lowered HIF-1α protein and HIF target gene expression (Nakayama *et al.* 2004). Accordingly, Le Moan *et al.* found decreased PHD3 mRNA levels as a result of a blunted HIF-response in p75 ko MEF cells. However, in our hands overexpression of the full-length p75 or the p75 intracellular domain did not affect hypoxic HIF-1α protein levels, suggesting that the p75/p75ICD cascade is not the major regulator of the hypoxia pathway in our cellular model (data not shown). To date, it remains unclear which other γ-secretase substrates besides APP is involved in the regulation of the HIF-signaling cascade.

To investigate the role of APP and APLP2 of the mouse brain *in vivo*, C57BL/6 wt, $APP^{fl/fl}$/APLP2 ko (Mallm *et al.* 2010) and Nex-Cre/$APP^{fl/fl}$/APLP2 cdko mice were exposed to 6 hours of 20% or 6% $O_2$ and transcript levels quantified. While EPO and VEGFA mRNAs were hypoxically induced by 20-fold and 4-fold, respectively, there was no difference between the three genotypes (data not shown). APP deletion efficiency in the cortex of Nex-Cre/$APP^{fl/fl}$/APLP2 cdko mice was approximately 70% on both mRNA and protein levels (data not shown). Of note, APP expression levels are very high in the brain and the remaining levels of APP might be sufficient to elicit a normal HIF response, making the results of these mouse models inconclusive.

Despite several *in vitro* and *in vivo* studies showing a direct link between Aβ and toxicity in FAD, the molecular nature of this interplay remains elusive (reviewed by Hardy and Selkoe 2002). An increasing number of reports provide evidence for non-physiological metal accumulation in the brains of patients with neurodegenerative diseases such as AD or PD (Barnham and Bush 2008). Iron and other transition metals can interact with Aβ, leading to increased Aβ deposition and to neuronal death through oxidative damage (Smith *et al.* 1997). A novel class of drugs target the abnormal metal accumulation, thereby reducing Aβ deposition and oxidative stress in the brain (Cuajungco *et al.* 2000; Doraiswamy and Finefrock 2004). Recently, M30 (5-(N-methyl-N-propargyaminomethyl)-8-hydroxyquinoline) and HLA20 (5-[4-propargylpiperazin-1-

ylmethyl]-8-hydroxyquinoline), two novel iron chelating drugs have been evaluated as therapeutic agents in AD (Zheng *et al.* 2005). Of note, iron chelators are well-known as PHD-inhibiting and HIF-activating compounds (Linden *et al.* 2003; Wang and Semenza 1993; Wanner *et al.* 2000). In particular, M30 has been shown to upregulate HIF and HIF-target genes in cortical neurons (Avramovich-Tirosh *et al.* 2010). This suggests that HIF-activation in the brain is beneficiary for patients suffering from AD and cognitive dysfunction, and that mechanisms impeding HIF-activation might be involved in the pathogenesis of the disease. Indeed, the classical HIF-targets EPO and VEGFA have been shown to improve cognitive function in mouse models when activated in the brain (Plaschke *et al.* 2008; Sargin *et al.* 2011). Additional studies to investigate if HIF-1$\alpha$ is able to rescue the age-dependent neurodegeneration in PSEN1/2 cdko mice (Saura *et al.*, 2004) would clarify many open questions. On the other hand, it has been suggested that hypoperfusion/hypoxia may mediate AD progression (Ogunshola and Antoniou 2009). Of note, a recent study showed elevated expression of HIF-1α in the brain microvasculature of an AD mouse model (Grammas *et al.* 2011). Furthermore, hypoxia has been shown to increase the expression of BACE1 (Sun *et al*., 2006; Zhang *et al*., 2007) and as a consequence the production of Ab (Webster *et al.*, 2002; Li *et al*., 2009; Kalaria *et al.,* 1993). In line with these data, we found lower BACE1 expression in the brain of PSEN1/2 cdko mice with decreased HIF-1$\alpha$. This suggests that decreased A$\beta$ production after PSEN1/2 ablation might be at least partly mediated vie HIF and BACE1. Furthermore, BACE1 was described to be an AICD target gene (von Rotz *et al.*, 2004). It is not clear if AICD regulates BACE1 and HIF independently or if AICD is regulating HIF that in turn regulates BACE1.

Intriguingly, PSEN1/2-deficient MEFs that were reconstituted with mutations originally identified in AD patients displayed a differential response to hypoxia. While the most severe $\gamma$-secretase loss-of-function mutations ($\Delta$E9, L199P) did not rescue HIF transcriptional activity, other mutations with mostly normal $\gamma$-secretase activity (A246E) showed a hypoxic response comparable to the wt response, confirming the importance of $\gamma$-secretase enzymatic activity for HIF regulation. Taking into consideration that FAD is a multifactorial progressive neurodegenerative disease, these results suggest that

HIF-1α protein levels and HIF transcriptional activity in the brain of AD patients deserve more clinical attention.

**Acknowledgements**

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# 4 Unpublished Data

## 4.1 Materials and Methods

*Plasmids and chemicals.* Full-length p75 and p75-ICD were kind gifts of Moses V. Chao (Skirball Institute, New York, USA). The cell-permeable proteasomal inhibitor MG-132 (Carbobenzoxy-L-leucyl-L-leucyl-L-leucinal) and was purchased from Calbiochem (Nottingham, UK). The specific γ-secretase inhibitor N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT), 5-aza-2-deoxycytidine and Imatinib (Gleevec) were purchased from Sigma (Buchs, Switzerland).

*Cell lines and transfections.* All cells were grown in high-glucose Dulbecco's modified Eagle's medium (DMEM; Sigma, Buchs, Switzerland). For hypoxic exposure, cells were cultured in a gas-controlled glove box (Invivo$O_2$ 400, Ruskinn Technologies, Leeds, United Kingdom). Transient transfections of MEFs were performed using Lipofectamine2000 (Invitrogen, Basel, Switzerland). HeLa, HepG2 and Hek293T cells were transiently transfected using the polyethylenimine (Polysciences, Warrington, PA, USA) method as described previously (Stiehl *et al.* 2006). For PION knock-down experiments, HepG2 and Hek293 cells were transfected with 100 nM siRNA oligonucleotides (Stealth®, Invitrogen) using Lipofectamine 2000 (Invitrogen).

*Mammalian-two-hybrid assay.* Mammalian two-hybrid assays were performed using the mammalian Matchmaker system (Clontech, BD Biosciences, Heidelberg, Germany) as described previously (Balamurugan *et al.* 2009). Cloning of G4-DBD-PSEN1 constructs was performed using Gateway technology (Invitrogen). HeLa cells were transiently co-transfected with 1.5 μg of G4-DBD- and 1.5 μg of VP16-AD fusion protein vectors together with 500 ng of firefly luciferase vector pGRE5xE1b and 20 ng of pRL-SV40. Total transfected DNA amounts were equalized in each experiments using the corresponding empty vector. Luciferase reporter gene activities were determined using the dual-luciferase reporter assay system (Promega, Madison, WI, USA).

*Immunoblotting.* Immunoblotting was performed as described previously (Balamurugan *et al.* 2009). Primary antibodies used were mouse anti-APP, clone 22C11 (Millipore, Zug, Switzerland, MAB348SP), anti-mHIF-1α (Novus Biologicals, NB100-479, Cambridge, UK), mouse anti-hHIF-1α (BD Transduction Laboratories, 610958), rabbit anti-p75 (Millipore, 07-476), mouse anti-β-actin (Sigma). Horseradish peroxidase coupled secondary anti-mouse and anti-rabbit antibodies were purchased from Pierce (Lausanne, Switzerland). Chemiluminescence detection was performed using Supersignal West Dura (Pierce) and signals were recorded with a charge-coupled device camera (Lightimager LAS-4000mini, Fujifilm, Dielsdorf, Switzerland).

*Protein extraction of mouse brains*. Brain sections were homogenized in Solution D and the first phase separation was performed following the RNA-extraction protocol described by (Chomczynski and Sacchi 1987). Protein was precipitated from the organic phase according the TRIZOL protein extraction protocol (Invitrogen). Final pellet was dissolved in 1% SDS followed by ultrasonication.

*RNA extraction and quantification.* Total RNA from cells or from the mouse brains was extracted as described previously (Wollenick *et al.* 2011). First-strand cDNA synthesis was performed with 1.5 μg RNA using affinity script reverse transcriptase (RT) enzyme (Stratagene, Amsterdam, Netherlands). mRNA expression levels were quantified by real-time quantitative (q) PCR using a SybrGreen qPCR reagent kit (Sigma) and a MX3000P cycler (Stratagene). Initial template concentrations of each sample were calculated by comparison with serial dilutions of a calibrated standard. To control for equal input levels, ribosomal protein S12 mRNA was determined and data were expressed as relative ratios.

### 4.2 Results

*PSEN1 regulates HIF-1α protein transcription or translation and interacts with HIF-1α*

To further analyze the PSEN-dependent regulation of HIF-1α, interference with HIF-1α protein degradation was eliminated. Therefore the proteasomal inhibitor MG132 was

applied to PSEN1/2 wt and ko MEFs and HIF-1α protein accumulation was analyzed after exposure to 20% or 0.2% $O_2$ for 16 hours. Both normoxic and hypoxic HIF-1α protein levels were lower in PSEN1/2 ko MEFs compared to the wt controls (Fig 1A), suggesting that HIF-1α protein is transcriptionally and/or translationally regulated by PSEN1/2.

Mammalian two-hybrid experiments were performed to investigate whether PSEN regulates the translation of HIF-1α by protein-protein interaction. Therefore, PSEN1 was fused to the Gal4 DNA binding domain and HIF-1α to the VP16 activation domain. Hypoxic Gal4 dependent luciferase activity was substantially higher when the Gal4-PSEN1 and VP16-HIF-1α fusion constructs were co-transfected compared to the transfections of either construct alone (8-fold over VP16-HIF-1α and 17-fold over Gal4-PSEN1) (Fig 1B). Co-transfection of Gal4-PHD2 with VP16-HIF1α was used as a positive control. Normoxic luciferase activities are low because VP16-HIF1α is constantly degraded. As published previously, PHD2 and Cdr2 do not interact and co-transfection of Gal4-PHD2 and VP16-Cdr2 expression vectors served as a negative control (Balamurugan *et al.* 2009).

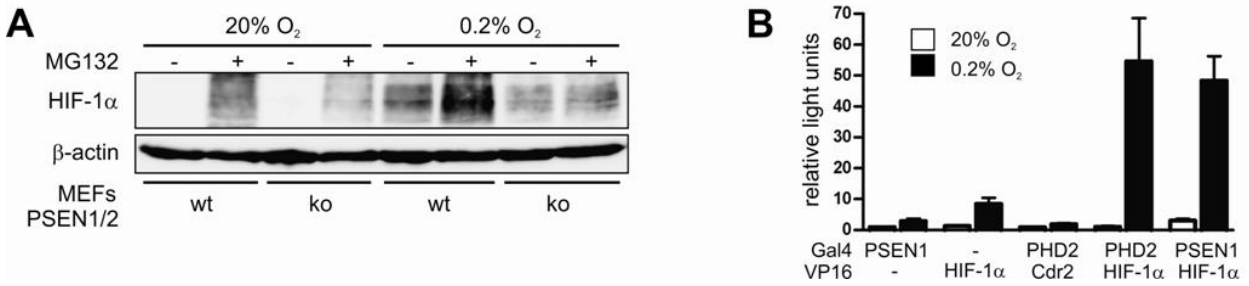



***Fig.1. PSEN1 interacts with HIF-1α and regulates HIF-1a protein transcription or translation***
***(A)*** *PSEN1/2 wt and ko MEFs were grown in the presence or absence of 10 μM MG132 for 1 hour before cultivating in 20% or 0.2% $O_2$ conditions for 16 hours. HIF-1α and β-actin protein levels were determined by immunoblotting.* ***(B)*** *HeLa cells were transiently transfected with Gal4-DNA-binding domain and VP16 activation domain fusion protein vectors and a Gal-4 response element-driven firefly luciferase reporter, as well as a Renilla luciferase control vector. Luciferase activities were determined 16 hours after exposure to 20% or 0.2% $_{O2.}$ Mean values ± SEM of three independent experiments performed in triplicates are shown. Relative normoxic luciferase activities after transfection with Gal4-PSEN1 were arbitrarily defined as 1.*

*Inhibition of Aβ generation via PION does not affect the hypoxia pathway*

A recent report showed that a newly discovered γ-secretase activating protein (GSAP or pigeon homolog (drosophila) PION) selectively increases Aβ production through a mechanism involving its interaction with both γ-secretase and APP (He *et al.* 2010). Gleevec (imatinib) that was previously found to inhibit Aβ formation without affecting Notch cleavage (Netzer *et al.* 2003) was shown to function through preventing PION interaction with APP. To test if PION-mediated specific APP cleavage is involved in the PSEN-dependent regulation of the hypoxia pathway, HepG2 and HEK293T cells were transiently transfected with siPION and cultured in 20% or 0.2% $O_2$ for 16 hours. PION knockdown had no effect on normoxic or hypoxic HIF-1α and PHD2 protein levels whereas PHD2 knockdown increased the normoxic HIF-1α protein accumulation in HepG2 cells (Fig. 2A). To confirm the results on transcript levels, siPION was transiently transfected in HepG2 cells and normoxic and hypoxic mRNA levels of PHD2, PHD3 and PION were analyzed by RT-qPCR (Fig. 2B). Whereas siPHD2 increased normoxic and hypoxic HIF-target genes, siPION did not affect constitutive expression or hypoxic induction of any of the analyzed HIF-target genes. To further investigate the involvement of specific Aβ inhibition on the hypoxia pathway, PSEN1/2 wt and ko MEFs were treated with 10 μM imatinib for 8 hours before incubating at 20% or 0.2% $O_2$ for 16 hours. PHD2, CAIX and HIF-1α mRNA levels were determined by RT-qPCR. Confirming our previous results, hypoxic PHD2 and CAIX accumulation was blunted in PSEN1/2 ko MEFs and constitutive HIF-1α levels were lower in the absence of PSEN1/2, but imatinib treatment did not have an effect (Fig. 2C). These results suggest that the PION/APP pathway is not involved in the PSEN dependent regulation of the HIF pathway in our models.

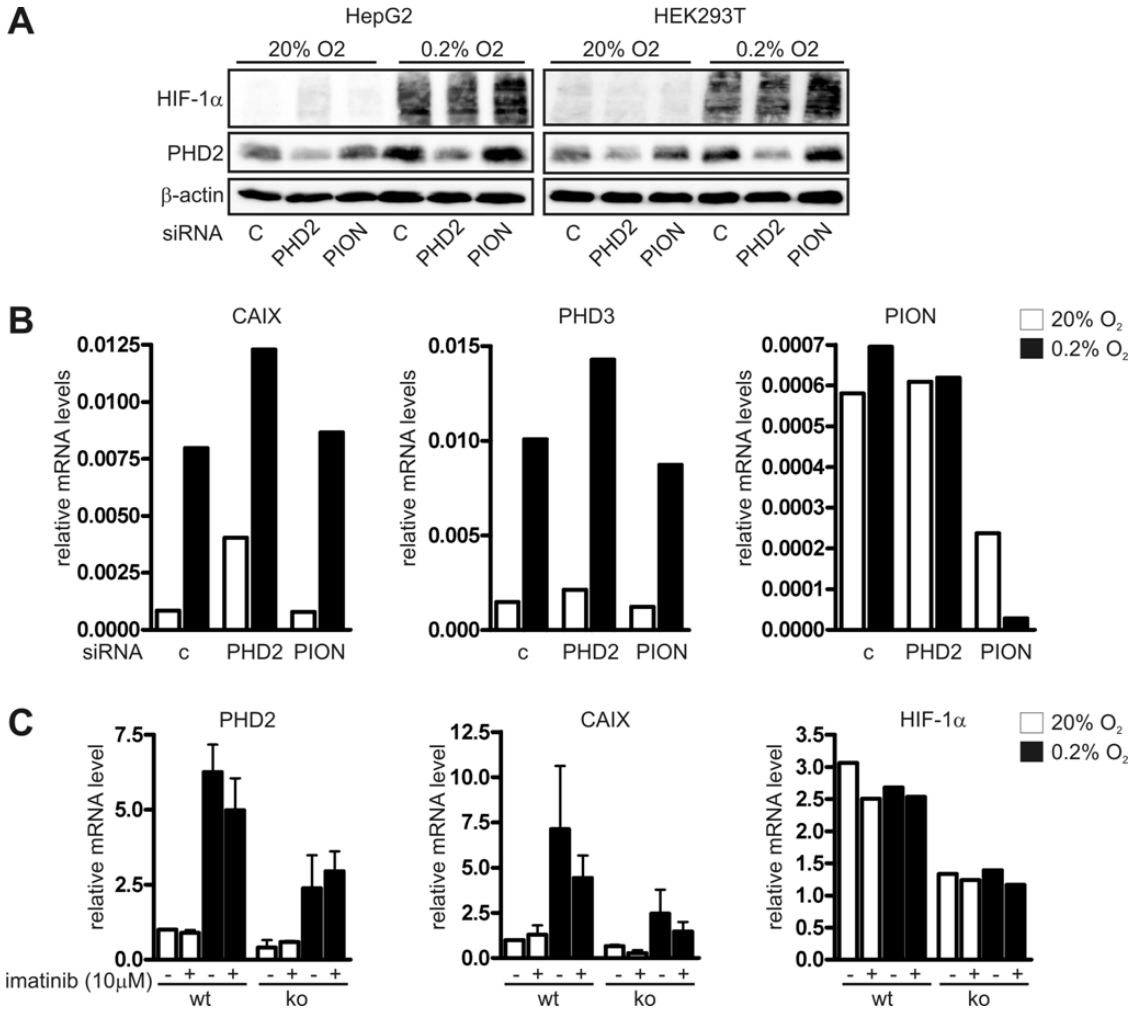



***Fig.2. GSAP is not involved in PSEN-dependent HIF-1α regulation****. HepG2 and Hek293 cells were transiently transfected with different siRNAs and cultured in 20% or 0.2% $O_2$ for 16 hours. HIF-1α , PHD2 and β-actin protein levels were analyzed by immunoblotting (**A**) and mRNA levels were determined by RT-qPCR (**B**). (**C**) HepG2 cells were treated with 10 μM imatinib for 8 hours before incubated in 20% or 0.2% $O_2$ for 16 hours. PHD2, CAIX and HIF-1α transcript levels were determined by RT-qPCR. Expression levels were normalized to the ribosomal protein S12 mRNA levels.*

*DNA methylation status is not regulated in a PSEN-dependent manner*

We further analyzed if the blunted HIF-1α response in PSEN1/2 ko MEFs could result from PSEN-dependent chromatin modifications. A recent report showed that the HIF-1α promoter contains a hypoxia response element that is normally repressed by methylation of a CpG dinucleotide within the core element. In colon cancer cell lines and in primary colon cancer specimens, aberrant demethylation of this element is frequently found, enabling binding of the HIF-1α to its own promoter resulting in auto-transactivation of HIF-1α expression (Koslowski *et al.* 2010). To test if PSEN dependent

regulation of the HIF-pathway could involve aberrant DNA methylation, PSEN1/2 wt and ko cells were treated with 1 μM 5-aza-2-deoxycytidine (5-aza-dC) for 72 hours before exposing to 20% or 0.2% $O_2$ for 16 hours. Transcript levels of HIF-1α and CAIX were analyzed by RT-qPCR. DNA demethylation by 5-aza-2-dC did not affect HIF-1α or CAIX transcript levels in MEFs. These data suggest that CpG demethylation does not affect hypoxic gene regulation in MEFs.

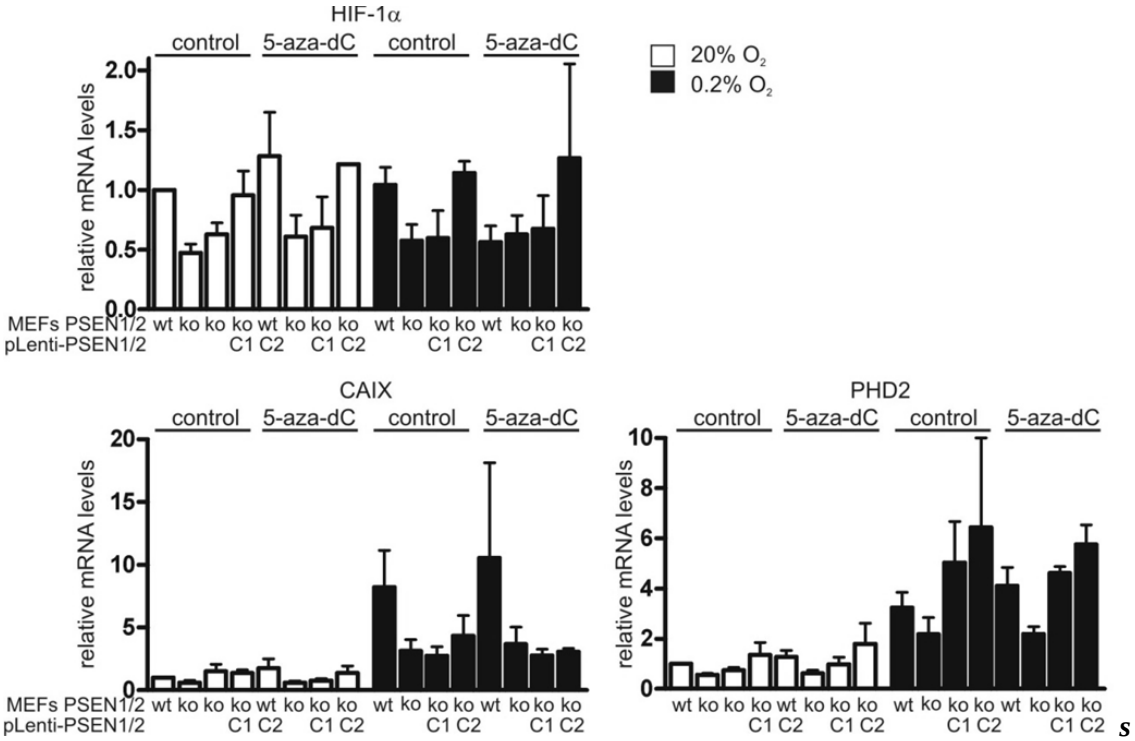



***Fig.3. 5-aza-dC treatment does not increase hypoxia-induced gene expression in PSEN MEFs.*** *To induce genomic DNA demethylation, cells were cultured in medium supplemented with 1μM 5-aza-2-deoxycytidine for 72 hours before incubating exposing to hypoxia. HIF-1α, CAIX and PHD2 mRNA levels were quantified by RT-qPCR after 16 hours exposure to 20% or 0.2% $O_2$. mRNA levels were normalized to expression levels of the ribosomal protein S12. Data is shown as values ± SEM of three independent experiments.*

*p75 cleavage is not altered in hypoxia nor affects HIF-1α levels*

A recent report by (Le Moan *et al.* 2011) showed that γ-secretase activity is increased in hypoxia by an unknown mechanism, leading to increased HIF-1α stability. Even though PSEN1/2 were not investigated, the authors found that genetic ablation of the γ-secretase substrate p75 neurotrophin receptor reduced hypoxic HIF-1α stabilization. Their findings were explained through decreased Siah2 abundance after p75

abrogation. Siah2 has been proposed previously to negatively regulate PHD1 and PHD3, and decreased Siah2 hence would lead to lowered HIF-1α protein and HIF target gene expression (Nakayama *et al.* 2004). Accordingly, Le Moan *et al.* found decreased PHD3 mRNA levels as a result of a blunted HIF-response in p75 ko MEF cells. However, we did not observe increased p75 cleavage different cell lines after exposure to hypoxia (Fig 4A). Furthermore, overexpression of full-length p75 or the p75 intracellular domain (p75ICD) did not affect hypoxic HIF-1α protein levels, suggesting that the p75/p75ICD cascade is not the major regulator of the hypoxia pathway in our cellular models (Fig 4).

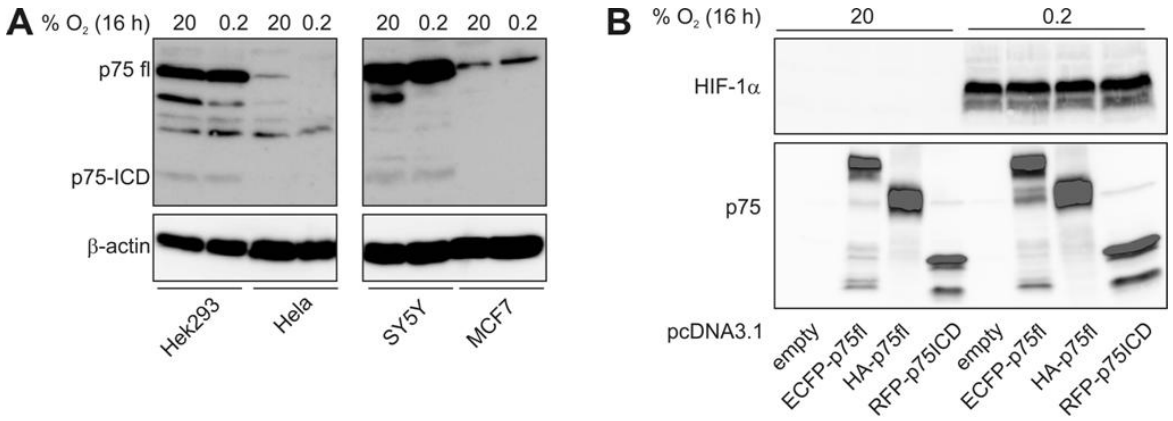
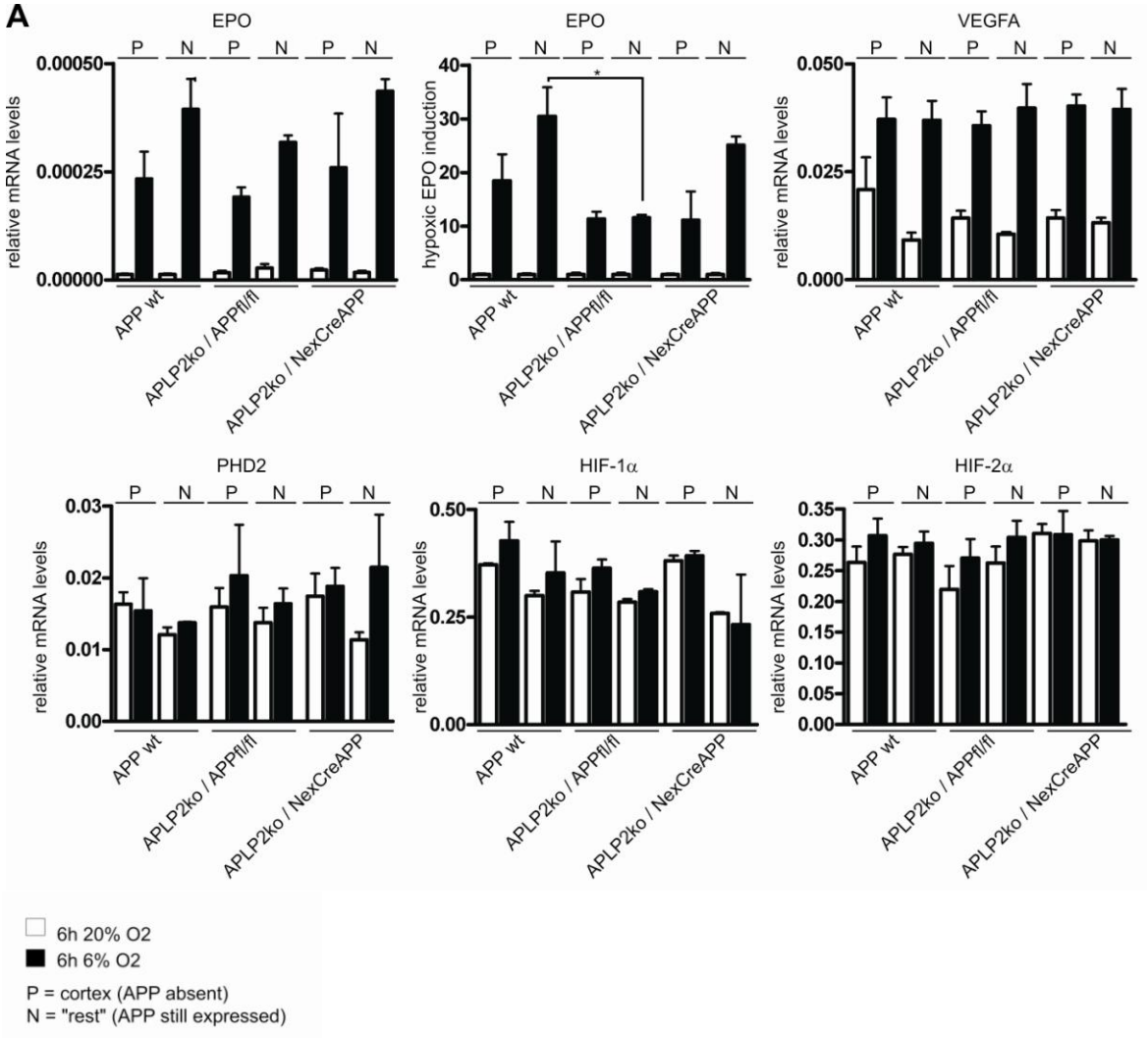



***Fig.4. p75 cleavage is not changed in hypoxia or affects HIF-1α.*** ***(A)*** *Different cell lines were exposed to 20% or 0.2% $O_2$ for 16 hours and p75 protein levels were analyzed by immunoblotting.* ***(B)*** *HeLa cells were transiently transfected with full length and p75-ICD. HIF-1α and p75 protein levels were analyzed by immunoblotting after 16 hours exposure to 20% or 0.2% $O_2$.*

*Brain specific conditional APP/APLP2 ko does not affect the HIF pathway*

Since hypoxic HIF-1α accumulation was blunted in APP ko and even further decreased in APP/APLP2 ko MEFs, we sought to analyze the involvement of APP and APLP2 *in vivo*. APP wt, APLP2ko/APPfl/fl and APLP2/APPfl/fl/NexCre mice (Ulrike Müller, Heidelberg) and exposed for 6 hours to 20% or 6% $O_2$ respectively. Since the NexCre specific APP knockdown was shown to be more efficient in the cortex (70%) than in the rest of the brains (Goebbels *et al.* 2006), the cortex (P) was dissected from the rest of the brain (N) and analyzed separately. While EPO and VEGFA mRNAs were hypoxically induced by 20-fold and 4-fold, respectively, there was no difference between the three genotypes (Fig 5A). APP deletion efficiency in Nex-Cre/APP$^{fl/fl}$/APLP2 cdko

mice was approximately 70% on both mRNA (Fig 5B) and protein levels (Fig 5C) in the (P) sections and 30% in the (N) sections. Of note, APP expression levels are very high in the brain and the remaining levels of APP might be sufficient to elicit a normal HIF response, making the results of these mouse models inconclusive. Furthermore, we cannot exclude that the decreased HIF-1α levels in the APP ko and APP/APLP2 dko MEF cell lines are not due to clonal variability of the different cell lines.



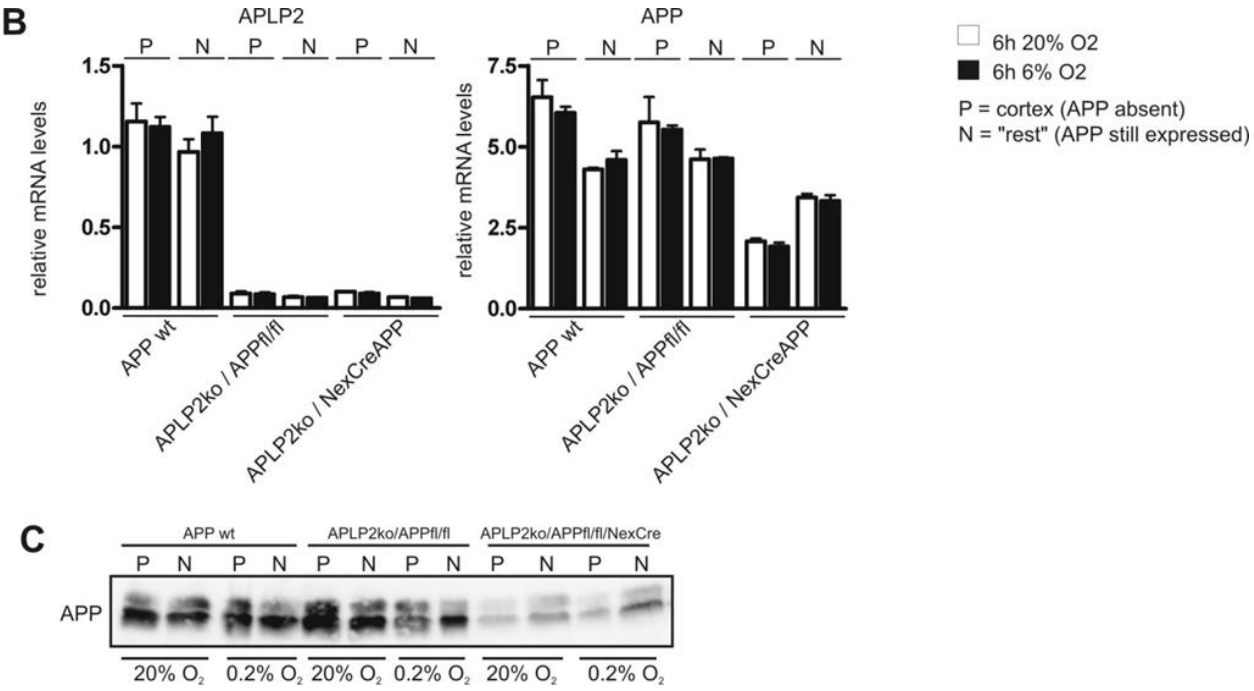



***Fig.5. Normal hypoxic response in the brain of conditional APP/APLP2 ko mice.*** *APP wt, APLP2ko/APPfl/fl and APLP2ko/APPfl/fl/NexCre mice were exposed to 20% or 6% $O_2$ for 6 hours and total RNA (**A** and **B**) and protein (**C**) was extracted from the Cortex (P) and the rest of the brain (N). EPO, VEGFA, PHD2, HIF-1$\alpha$, HIF-2$\alpha$, APLP2 and APP transcript levels were quantified by RT-qPCR and normalized to the expression of ribosomal protein S12 mRNA. Data is shown as mean values ± SEM of three different mice (**A** and **B**). APP protein levels were analyzed by immunoblotting (**C**).*

# 5 Discussion

*The role of PSEN in the regulation of the oxygen-sensing pathway*

We could show that PSEN affects HIF-signaling on more than one level. MEFs lacking PSEN1/2 showed increased constitutive FKBP38 and concomitantly decreased PHD2 protein levels. These findings are in line with a previous report that PSEN1/2 increases FKBP38 protein levels (Wang *et al.* 2005) and with our previous results showing that FKBP38 negatively regulates PHD2 protein abundance (Barth *et al.* 2007). Together with PSEN enhancer (Pen-2), nicastrin, and anterior pharynx 1 (Aph-1), PSENs form the core of the γ-secretase complex that cleaves a variety of type I transmembrane proteins (De Strooper *et al.* 1999; Parks and Curtis 2007). Several studies implied that PSEN also have γ-secretase independent functions (reviewed by Wakabayashi and De Strooper 2008). The PSEN1/2-dependent regulation of FKBP38 was shown to independent of the γ-secretase function since DAPT treatment did not affect the PSEN-FKBP38 interaction an neither FKBP38 protein levels (Wang *et al.* 2005). Accordingly, we showed that γ-secretase inhibition did not affect neither FKBP38 nor PHD2 protein levels. PHD2 protein levels were substantially downregulated, FKBP38 protein levels were only slightly upregulated, suggesting that additional mechanisms contribute to the decreased PHD2 levels. Consistently, PHD2 mRNA levels were significantly downregulated in PSEN1/2 deficient MEFs as well as in the cortex of forebrains-specific conditional PSEN1/2 ko mice. Since PHD2 is considered to be the main oxygen sensor that promotes HIF-α degradation in normoxia and we and others previously showed that slight changes in PHD2 protein levels affect HIF-1α abundance under normoxic as well as hypoxic conditions (Ginouvès *et al.* 2008; Henze *et al.* 2010; Stiehl *et al.* 2006), we expected an increased hypoxic response in the PSEN1/2 ko MEFs. Surprisingly, we found a decreased HIF response in PSEN1/2 deficient MEFs. Since PHD2 is a direct target of HIF-1α, these findings might provide the additional mechanism involved in the substantial downregulation of PHD2 in the absence of PSEN1/2.

Even though HIF-1α and HIF-2α enhance the expression of a specific set of target genes, they have as well overlapping functions and a vast majority of hypoxia-induced genes are regulated by both HIF-1 and HIF-2. One prominent difference between

HIF-1α and HIF-2α is observed in their spatial expression patterns. HIF-1α is ubiquitously expressed, whereas HIF-2α expression is highest in alveolar epithelial cells in the lung but also in endothelial cells of various tissues, such as brain, heart, kidney and liver (Ema *et al.* 1997; Flamme *et al.* 1997). While HIF-1α and HIF-2α have been used interchangeably for *in vitro* hydroxylation analysis (Epstein *et al.* 2001; Jaakkola *et al.* 2001; Lando *et al.* 2002), there is growing evidence that they play distinct roles, especially during tumor progression (reviewed in Keith *et al.* 2012). To investigate if HIF-2α is also regulated in a PSEN-dependent manner in MEFs, constitutive HIF-2α mRNA expression was quantified by RT-qPCR in PSEN1/2 wt and ko MEFs. While HIF-1α mRNA levels were reduced by almost 50% in the absence of PSEN1/2, surprisingly HIF-2α mRNA levels were found to be even higher (data not shown). Nevertheless, whereas lentiviral silencing of HIF-1α completely abolished the hypoxic induction of all analyzed HIF-target genes, HIF-2α knockdown did not affect any of the analyzed genes, independent of oxygen exposure. These data suggest, that HIF-1α is the main regulator of the hypoxic response in MEFs. These results are in line with a publication from Park *et al.,* where hypoxia-induced gene expression was found to occur solely through the action of HIF1α in MEFs (Park *et al.* 2003). HIF-2α was shown to be expressed at constant levels regardless of oxygenation and to be primarily localized to the cytoplasm. HIF-2α was not capable of stimulating transcription of known HIF-1 target genes in response to hypoxia. Nevertheless, HIF-2α is known to play a critical role in the brain. Consistently, HIF-1α and HIF-2α mRNA were decreased in the cortex of PSEN1/2 conditional double knock-out brains, suggesting that PSEN1/2 regulate both isoforms. Further studies are needed to pin down the role of HIF-1 versus HIF-2 in the PSEN-dependent regulation of the oxygen sensing pathway.

During the progression of our study, Gasperi *et al.* showed that PSEN1 ko MEFs display impaired induction of HIF-1α following stimulation with hypoxia mimetics, insulin and calcium chelators (De Gasperi *et al.* 2010). These authors attributed their findings to a shorter half life of HIF-1α in PSEN1-deficient MEFs. However, using PSEN1/2-deficient MEFs, we could not find any decrease in HIF-1α protein stability. Two main differences between our experiments and those performed by Gasperi *et al.* might explain this

discrepancy: first, we conducted our analysis in double-ko MEFs; and second we measured HIF-1α decay following a controlled hypoxic accumulation since HIF-1α is normally not detectable under normoxic conditions.

Using mammalian two hybrid assays, we found that PSEN1 interacts with HIF-1α. Since HIF-1α protein stability was not decreased by the absence of PSEN1/2, PSEN1/2 might rather be involved in the regulation of transcription and translation of HIF-1α. PSEN1/2 have been shown to be essential for maturation and transport of integrin-β1 in the ER-Golgi compartment (Zou *et al.* 2008) which raises the possibility that HIF-1α regulatory function of PSEN1/2 localizes to the ER membrane. However, we currently do not know how PSEN1/2 affects HIF-1α translation.

*Role of APP/AICD cleavage cascade in the regulation of HIF*

NICD and AICD are two major γ-secretase dependent intracellular cleavage products (derived from the Notch and APP precursors, respectively) and NICD has been shown previously to potentiate the HIF response in neuronal and myogenic cells (Gustafsson *et al.* 2005). However, in the cell models used in this study AICD but not NICD regulated HIF-1α and HIF target gene expression. The function of AICD remains incompletely understood but it has been shown to be present in nuclear complexes involved in nuclear signaling (Cao and Sudhof 2001; von Rotz *et al.* 2004). Of note, several genes regulated by AICD have been identified, including KAI1 (Baek *et al.* 2002), glycogen synthase 3β (Kim *et al.* 2003), APP and BACE (von Rotz *et al.* 2004). Our results suggest that HIF-1α might represent a novel AICD target gene, but further mechanistic experiments need to be performed to corroborate this hypothesis. It is for instance not clear, whether AICD directly or via the interaction with other proteins localizes to the HIF-1α promoter. Chromatin immunoprecipitation (ChIP) experiments for AICD on the HIF-1α promoter would be needed to investigate this hypothesis.

In line with our results in AICD overexpressing cells, hypoxic HIF-1α accumulation as well as hypoxic induction of HIF-target genes was blunted in APP/APLP2 ko MEFs and to a lesser extent in APP ko MEFs. APP, APLP1 and APLP2 have been shown to be partially redundant and the APP/APLP-ICDs were also found to be involved in

transcription (Eggert *et al.* 2004; Pardossi-Piquard *et al.* 2005; Scheinfeld *et al.* 2002; Walsh *et al.* 2003). A NPTY motif in the C-termini of APP, APLP1 and APLP2 interacts with several phosphotyrosin/protein binding (PTB) proteins, including the FE65 protein family (Bressler *et al.* 1996; Guenette *et al.* 1996; McLoughlin and Miller 1996; Tanahashi and Tabira 1999). To corroborate our results, we exposed wt, APLP2ko/APP$^{fl/fl}$ and the conditional Nex-Cre/APP$^{fl/fl}$/APLP2 cdko mice to 6 hours 20% or 6 % $O_2$ respectively and extracted mRNA and protein from two different brain regions that are expected to have different efficiencies of APP deletion. While EPO and VEGFA mRNAs were hypoxically induced 20-fold and 4-fold, respectively, there was no difference between the three genotypes. APP deletion efficiency in the cortex of mice was approximately 70% on both mRNA and protein levels Of note, APP expression levels are very high in the brain and the remaining levels of APP might be sufficient to elicit a normal HIF response, making the results of these mouse models inconclusive. Furthermore, we cannot exclude that the decreased HIF-1$\alpha$ levels in the APP ko and APP/APLP2 dko MEF cell lines are not due to clonal variability of the different cell lines.

A recent report by (Le Moan *et al.* 2011) showed that $\gamma$-secretase activity is increased in hypoxia by an unknown mechanism, leading to increased HIF-1$\alpha$ stability. Even though PSEN1/2 were not investigated, the authors found that genetic ablation of the $\gamma$-secretase substrate p75 neurotrophin receptor reduced hypoxic HIF-1$\alpha$ stabilization. Their findings were explained through decreased Siah2 abundance after p75 abrogation. Siah2 has been proposed previously to negatively regulate PHD1 and PHD3, and decreased Siah2 hence would lead to lowered HIF-1$\alpha$ protein and HIF target gene expression (Nakayama *et al.* 2004). Accordingly, Le Moan *et al.* found decreased PHD3 mRNA levels as a result of a blunted HIF-response in p75 ko MEF cells. However, in our hands overexpression of the full-length p75 or the p75 intracellular domain did not affect hypoxic HIF-1$\alpha$ protein levels, suggesting that the p75/p75ICD cascade is not the major regulator of the hypoxia pathway in our cellular models.

Intriguingly, PSEN1/2-deficient MEFs that were reconstituted with mutations originally identified in AD patients displayed a differential response to hypoxia. While the most

severe γ-secretase loss-of-function mutations (ΔE9, L199P) did not rescue HIF transcriptional activity, other mutations with mostly normal γ-secretase activity (A246E) showed a hypoxic response comparable to the wt response, confirming the importance of γ-secretase enzymatic activity for HIF regulation.

*Additional mechanisms of PSEN-dependent regulation of HIF-1α*

Recently, a novel γ-secretase activating protein (GSAP or PION) was identified which selectively increased Aβ production through a mechanism involving its interaction with the APP-CTF (He *et al.* 2010). Knockdown of PION in a mouse model of AD reduced Aβ levels and plaque development. Since PION represents a new type of γ-secretase regulators that affects some of its substrates (like APP) but not others (like Notch), we sought to investigate whether PION could play a role in the PSEN-dependent regulation of the hypoxia pathway. Knock-down experiments in different cell lines did not show any affect on hypoxic HIF-1α protein levels and neither on the hypoxic induction of HIF-target genes. Since GSAP regulates the production of Aβ but not of the concomitantly formed AICD that we found to be involved in the regulation of HIF-1α, these results are not surprising.

A recent report showed that the HIF-1α promoter harbors a hypoxia response element that is normally repressed by methylation of a GcG dinucleotide located in the core element (Koslowski *et al.* 2010). Since HIF-1a mRNA is regulated in a PSEN-dependent manner, we hypothesized that the blunted HIF-1α response in PSEN1/2 ko MEFs could result from PSEN-dependent chromatin modifications. Treating PSEN1/2 wt and ko MEFs with the DNA-demethylation agent 5-aza-dCT did not affect HIF-1α, CAIX or PHD2 mRNA levels in normoxia or hypoxia. These data suggest that CpG demethylation does not affect hypoxic gene regulation in MEFs.

*The role of hypoxia in Alzheimer's disease*

The brain is one of the most susceptible organ to oxygen deprivation. Only a few minutes of severe hypoxia initiates significant dysfunction and can ultimately result in cell death (Siesjo 1988). Oxygen delivery to the aging brain, to cells and tissues gets

impaired, thereby increasing the susceptibility to neuronal damage. In addition, cellular adaptation to hypoxia is significantly compromised with increasing age and ischemia has more severe effects in old versus young patients (Kolb 2003; Yager *et al.* 2006).

Alzheimer's disease is a multifactorial disorder in which both genetic and environmental factors contribute to disease progression. Genetic predisposition though mutations in APP or PSEN are responsible for only 5% of all AD cases and result in familial early onset AD (Coppede *et al.* 2006). Exposure to other pathogenic conditions, including chronic inflammation, cerebrovascular disease and hypoxia/ischemia episodes could be important contributing factors (Jellinger and Attems 2005; Aliev *et al.* 2002; Coppede *et al.* 2006).

*In vitro*, simultaneous treatment of cortical neurons with Aβ and hypoxia drastically increases the number of apoptotic cells compared to Aβ treatment alone (Egashira *et al.* 2002). Nevertheless, the molecular mechanisms that link hypoxia to AD progression are incompletely understood. It has been shown that hypoxia/ischemia and hypoperfusion upregulates APP at both the mRNA and protein levels and leads to subsequent Aβ accumulation (Jendroska *et al.* 1997; Shi *et al.* 2000). However, we did not find any evidence of increased APP mRNA or protein levels in hypoxia, neither in the cortex of wt or APLP2 ko mice, nor in the cell lines analyzed. There is evidence that short periods of hypoxia enhances the Aβ-induced expression of the pro-inflammatory markers cyclooxygenase-2 (COX-2) as well as PSEN1, hence accelerating the inflammatory neuropathology that characterized AD brains (Bazan and Lukiw 2002). Again, we did not observe any changes of PSEN1, PSEN2 in hypoxia, whereas COX-2 expression was slightly increased in the analyzed cell lines or mouse tissues. This suggests that the hypoxic induction of AD-related genes is highly cell type and context dependent and further studies are needed to better understand the role of hypoxia in AD.

Since over a decade, there is evidence that iron chelators such as desferroxamine, protect cultured cells from Aβ (Schubert and Chevion 1995) and inhibit AD progression in patients (Mclachlan *et al.* 1991). Of note, iron chelators are well-known as PHD-inhibiting and HIF-activating compounds (Linden *et al.* 2003; Wang and Semenza 1993; Wanner *et al.* 2000). Another metal chelator, clioquinol, was concurrently shown to reverse AD pathology in mice (Mclachlan *et al.* 1991) and induce *in vitro* HIF-1α

expression (Soucek *et al.* 2003). Recently, M30 (5-(N-methyl-N-propargyaminomethyl)-8-hydroxyquinoline) and HLA20 (5-[4-propargylpiperazin-1-ylmethyl]-8-hydroxyquinoline), two novel iron chelating drugs have been evaluated as therapeutic agents in AD (Zheng *et al.* 2005). In particular, M30 has been shown to upregulate HIF and HIF-target genes in cortical neurons (Avramovich-Tirosh *et al.* 2010). Soucek *et al*. showed that low levels Aβ directly induce HIF-1α expression and activity *in vitro* that protects cells from Aβ-induced neurotoxicity. In line with this, HIF-1 levels were found to be reduced in brains from AD compared to age-matched controls (Liu *et al.* 2008). To further support the beneficial effects of HIF in AD progression, EPO was found to be both necessary and sufficient to prevent Aβ-induced apoptosis in the early and later stages of neurodegeneration (Chong *et al.* 2005). The underlying mechanism involves the expression and translocation of NF-κB p65. However, the precise role of the pathway and whether it is beneficial or detrimental remains a matter of debate. Particularly since HIF-signaling pathways have been implicated in cell death as well as cell survival, depending on the cell type and condition (Vangeison *et al.* 2008). Notably, Zhang *et al*. showed that BACE expression is upregulated during acute hypoxia by HIF-1, thus promoting Aβ production in neuroblastoma cells (Zhang *et al.* 2007). Furthermore HIF-1 has been shown to bind to the APH-1 promoter that leads to subsequent γ-secretase-mediated Aβ and Notch generation during hypoxia (Wang *et al.* 2006).

Thus, the overall contribution of hypoxia to the pathogenesis of AD remains elusive and may greatly depend on whether it is the cause or the consequence of the disease progression. Further studies are needed to understand the role of hypoxia in the context of neurodegeneration since the modulation of HIF-signaling may represent new hope for the development of novel AD therapies.

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# Project II: Onconeuronal Cerebellar Degeneration-Related Antigen Cdr2 as a Novel Diagnostic Marker for Renal Cancer

## 1 Introduction

### 1.1 The Cerebellar Degeneration-Related Onconeural Antigen Cdr2

The cerebellar degeneration-related antigen Cdr2 was discovered in connection with the paraneoplastic cerebellar degeneration (PCD) syndrome in cancer patients with anti-tumor immunity. PCD is mainly found in patients with breast and ovarian tumors and is characterized by the presence of specific auto-antibodies against Cdr2 (also termed anti-Yo) (Anderson *et al.* 1988). Corradi et al (Corradi *et al.* 1997) analyzed the first PCD tumors for the expression of different Cdr isoforms (Cdr 1-3). Whereas all three Cdr transcripts could be detected in the cerebellum, only Cdr2 could be detected in the PCD ovarian tumors. Analysis of Cdr2 protein expression in gynecological tumors obtained from neurologically normal cancer patients revealed that five out of nine representative ovary tumors expressed a 52 kDa protein, migrating at the same position as Cdr2 from Purkinje cells. Cdr2 expression was as well found in breast tumor patients without PCD. These findings suggest an ectopic expression of Cdr2 in a large number of gynecological tumors, independent of the presence of paraneoplastic neurological degeneration (Darnell and Albert 2000).

Cdr2 has two family members, Cdr1 and Cdr3. Whereas Cdr2 and Cdr3 show 44% protein homology, Cdr2 and Cdr1 overlap only to 13%. We generated a polyclonal anti-Cdr2 antibody by immunizing rabbits with the synthetic peptide PEYKALFKEIFSCIK, a conserved C-terminal part of Cdr2 (aa420-434) and does not recognize the other isoforms (licensed to Novus, NBP2-10509).

#### *1.1.1 Cdr2 Expression*

While Cdr2 mRNA is ubiquitously expressed, protein expression is restricted to the brain and testis (Corradi *et al.* 1997). Sequence analysis of the brain and spleen Cdr2 cDNA showed 100% identity, suggesting a tissue-specific post-translational regulation mechanism. Immunohistochemical analysis showed that Cdr2 protein is expressed in cerebellar Purkinje neurons, brainstem neurons and spermatogonia (Corradi *et al.*

1997). Neoplastic Cdr2 expression in gynecological tumors was detected in five out of nine representative ovary tumors as well as in some breast tumor patients without PCD (Darnell *et al.* 2000; Darnell and Albert 2000).

### *1.1.2 Physiological Function and Regulation*

The physiological function of Cdr2 is incompletely understood. Cdr2 was found to interact with c-Myc in yeast and *in vitro.* (Okano *et al.* 1999). Immunohistochemical analysis of rat brain sections showed a significant co-localization of Cdr2 and c-Myc in the cytoplasm of purkinje neurons (Okano *et al.* 1999). Overexpression of Cdr2 was shown to lead to a redistribution of c-Myc into the cytoplasm, where it co-localized with Cdr2. Furthermore, Cdr2 attenuated c-Myc-dependent reporter gene expression. Additionally, the leucine zipper motif has been demonstrated to interact with cell cycle-related proteins (Sakai *et al.* 2004; Sakai *et al.* 2002) and with a protein kinase (Takanaga *et al.* 1998). More recently, Cdr2 was found to be regulated in a cell cycle dependent manner in cancer cells with highest protein levels during mitosis (O'Donovan *et al.* 2010). Cdr2 is phosphorylated after mitosis by the anaphase promoting complex/cyclosome (APC/C) and rapidly degraded by the proteasome. Loss of Cdr2 was found to lead to aberrant mitotic spindle formation, suggesting that Cdr2 acts in mitosis to regulate c-myc target genes in tumor cells.

### *1.1.3 The Role of Cdr2 in Paraneoplastic Neuronal Degradation*

PNDs are a diverse group of human neurological diseases that are associated with cancer anti-tumor immunity. By yet unknown mechanisms, tumors of mainly breast, ovarian or lung cancer patients express neuronal antigens that triggers an antitumor immune response. The neoplastic expression of these antigens suppresses tumor growth, but also develops in an autoimmune neurodegenerative disease. (Albert and Darnell 2004). The most commonly found tumor antigens include the nerve-terminal vesicle-associated proteins such as the breast cancer/stiff-person PND antigen amphiphysin (Folli *et al.* 1993), cytoplasmic signaling proteins such as the PCD antigen cerebellar degeneration-related-antigen Cdr2 (Corradi *et al.* 1997; Okano *et al.* 1999) and RNA-binding proteins that have various regulatory functions, such as the POMA

and Hu PND antigens neuro-oncological ventral antigen (NOVA) and Hu (Brennan and Steitz 2001; Dredge *et al.* 2001; Keene 1999).

Cdr2 is expressed in 60% of ovarian tumors and approximately in 25% of the breast tumors (Darnell *et al.* 2000). Yet not all of these patients develop neurodegenerative disorders (Peterson *et al.* 1992).

*Development of tumor immunity and pathogenesis of PND*

A model for the development of tumor immunity in paraneoplastic neurological disease is depicted in Figure 1 (Albert and Darnell 2004). PNDs are thought to be initiated when tumor cells (in blue) start expressing proteins (red star) that are normally restricted to neurons. The expression of such onconeural antigens triggers an anti-tumor immune response. Some of the antigens, like Cdr2, are also expressed in other immunoprivileged organs such as the testis. Even though the tumor antigen is identical to the neural antigen, it elicits an immune response that is not fully understood. Apoptotic tumor cells are phagocytosed by dendritic cells (purple) that migrate to the lymph nodes and activate antigen-specific $CD4^+$, $CD8^+$ and B cells. Activated CD8+ cells return to the tumor, where they induce apoptotic death triggering a positive-feedback loop that potentiates the immune response. At the same time, the antibodies and T cells raised against the tumor antigen are able to cross the blood brain barrier and attack neurons that physiologically express the antigen (Albert and Darnell 2004; Darnell and Posner 2003).

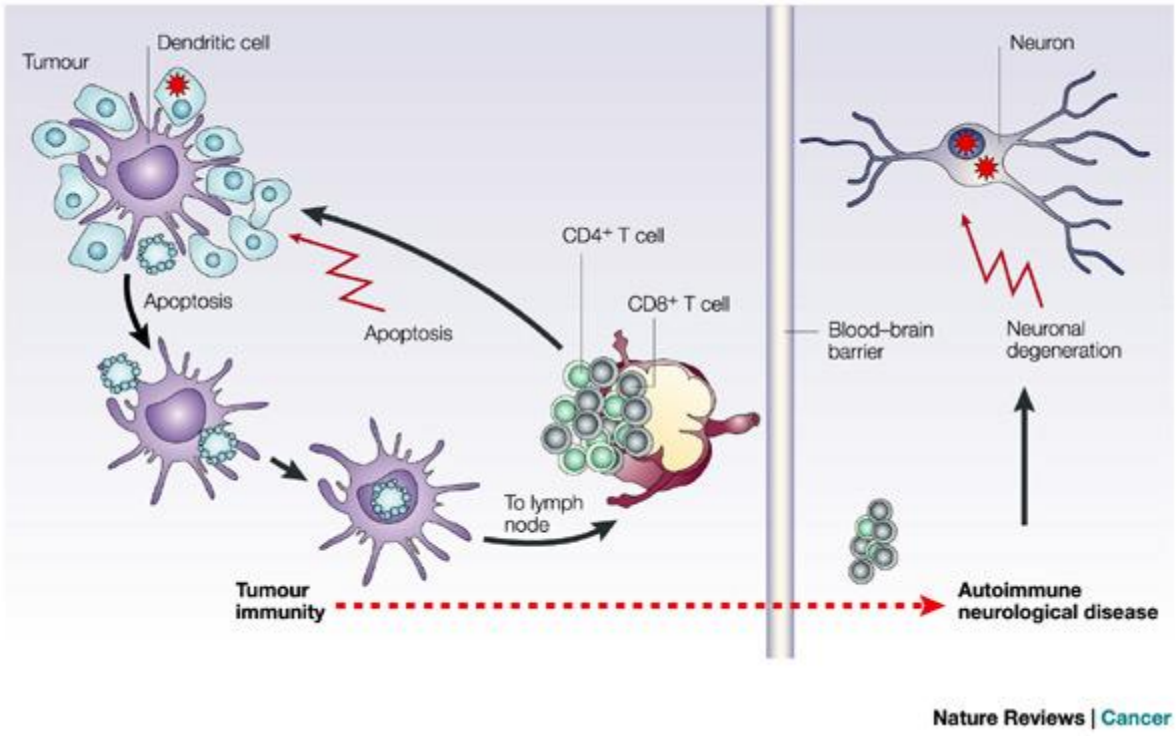



***Fig.1. Model for the development of tumor immunity in paraneoplastic neurological disorders from*** *(Albert and Darnell 2004). Paraneoplastic neurological degenerations are initiated when tumor cells (blue) express proteins (red star) that are normally only expressed in neurons. This elicits an immune response. The development of the neurological disease then involves B and T cells that cross the blood-brain-barrier (red dashed line). The recognition of neurons that normally express the PCD antigens leads to the development of neuronal degeneration.*

## 2 Original Publication

### Onconeuronal cerebellar degeneration-related antigen Cdr2 is strongly expressed in papillary renal cell carcinoma and leads to attenuated hypoxic response

Kuppusamy Balamurugan[1,3], Van-Duc Luu[2], Muriel R. Kaufmann[1], Verena S. Hofmann[1], Gunther Boysen[2], Sandra Barth[1,4], Mattia R. Bordoli[1], Daniel P. Stiehl[1], Holger Moch[2], Peter Schraml[2], Roland H. Wenger[1] and Gieri Camenisch[1]

[1]Institute of Physiology and Zürich Center for Integrative Human Physiology ZIHP, University of Zürich UZH, CH-8057 Zürich, Switzerland, [2]Department of Pathology, Institute of Surgical Pathology, University Hospital Zürich, CH-8091 Zürich, Switzerland

Correspondence: Gieri Camenisch, Institute of Physiology, University of Zürich, Winterthurerstrasse 190, CH-8057 Zürich, Switzerland. Tel: +41 (0)44 6355075; Fax: +41 (0)44 6356814; E-Mail: gieri.camenisch@access.uzh.ch

[3]Present address: Molecular Mechanisms in Development Group, Center for Cancer Research, National Cancer Institute, Frederick, MD 21702-1201, USA

[4]Present address: Ben May Department for Cancer Research, University of Chicago, Gordon Center for Integrative Sciences, Chicago, IL 60637, USA

**Running Title:** Cdr2 suppresses HIF function in papillary RCC

**Key words:** renal cell carcinoma, tumor immunity, paraneoplastic cerebellar degeneration, oxygen sensing

**Abstract**

The onconeuronal cerebellar degeneration-related antigen Cdr2 is associated with paraneoplastic syndromes. Neoplastic expression of Cdr2 in ovary and breast tumors triggers an autoimmune response that suppresses tumor growth by developing tumor immunity, but culminates in cerebellar degeneration when Cdr2-specific immune cells recognize neuronal Cdr2. We identified Cdr2 as novel interactor of the hypoxia-inducible factor (HIF) prolyl-4-hydroxylase PHD1 and provide evidence that Cdr2 might represent a novel important tumor antigen in renal cancer. Strong Cdr2 protein expression was observed in 54.2% of papillary renal cell carcinoma (RCC) compared to 7.8% of clear cell RCC and no staining in chromophobe RCC or oncocytoma. High Cdr2 protein levels correlated with attenuated HIF target gene expression in these solid tumors and Cdr2 overexpression in tumor cell lines reduced HIF-dependent transcriptional regulation. This effect was due to both attenuation of hypoxic protein accumulation and suppression of the transactivation activity of HIF-1$\alpha$. Papillary (p)RCC is known for its tendency to avascularity, usually associated with a lower pathological stage and higher survival rates. We provide evidence that Cdr2 protein strongly accumulates in pRCC, attenuates the HIF response to tumor hypoxia and may become of diagnostic importance as novel renal tumor marker.

**Introduction**

Hypoxia is a characteristic feature of the micro-environment of many tumor types, and contributes to malignant tumor behavior associated with therapy resistance and poor prognosis (Brown and Wilson, 2004; Pouysségur et al., 2006). Heterodimeric hypoxia-inducible transcription factors (HIFs) are master regulators of oxygen homeostasis and many HIF-target genes are involved in adaptive changes of cancer cells to their hypoxic micro-environment (Wenger, 2002; Wenger et al., 2005). On the molecular level, cells sense alterations in oxygen levels by oxygen-dependent HIF prolyl and asparaginyl hydroxylation which determines HIF-$\alpha$ protein stability and transactivation activity, respectively. Oxygen-dependent hydroxylation of two distinct HIF-$\alpha$ prolyl residues by the three HIF prolyl-4-hydroxylase domain proteins PHD1, 2 and 3 (alternatively termed HPH3/EGLN2, HPH2/EGLN1 and HPH1/EGLN3, respectively) is necessary for the interaction with the von Hippel-Lindau tumor suppressor protein (pVHL) that serves as recognition unit of a multiprotein ubiquitin E3 ligase and targets HIF-$\alpha$ for proteasomal degradation (Bruick and McKnight, 2001; Epstein et al., 2001; Ivan et al., 2002; Jaakkola et al., 2001; Maxwell et al., 1999). Compared with collagen prolyl-4-hydroxylase and mitochondrial cytochrome c oxidase, PHDs have a lower $O_2$ affinity that suits these enzymes to regulate HIF-$\alpha$ protein levels in response to a wide range of physiologically relevant $pO_2$ (Schofield and Ratcliffe, 2004). Hydroxylation of an asparaginyl residue in the transactivation domain of HIF-$\alpha$ by factor inhibiting HIF (FIH) blocks the interaction with the transcriptional co-activator p300 and thus impairs the induction of target genes by regulating the transactivation activity of HIF (Mahon et al., 2001).

Importantly, mass spectroscopic evidence for hydroxylation of ankyrin repeats in I$\kappa$B and NF-$\kappa$B family members as well as in ASB4, a suppressor of cytokine signaling, by FIH demonstrated that hydroxylation is not restricted to HIF-$\alpha$ subunits (Coleman et al., 2007; Ferguson et al., 2007). Although direct mass spectroscopic evidence for hydroxylation is missing, PHD1 and PHD3 have recently been shown to regulate I$\kappa$B kinase-$\beta$ (IKK$\beta$) and activating transcription factor-4 (ATF-4), respectively (Cummins et al., 2006; Koditz et al., 2007). Apart from mediating HIF-$\alpha$ protein stability, these data

indicate that PHDs might regulate additional oxygen-dependent signaling pathways. We identified Cdr2 as novel PHD1 interacting partner and comprehensive analysis on tissues and tumor cell lines demonstrated that abundant Cdr2 protein levels are a characteristic feature of papillary RCC and might lead to attenuated hypoxic response pathways in these tumors.

**Materials and Methods**

*Plasmids.* Cloning work was carried out using Gateway technology (Invitrogen, Basel, Switzerland). Cloning of PHD 1 to 3 vectors was previously described (Barth et al., 2007). Entry vectors were generated by cloning PCR fragments into *Nco*I/*Xho*I-digested pENTR4 (all restrictions enzymes were purchased from MBI Fermentas, Labforce, Nunningen, Switzerland). Full-length Cdr2 (residues 1 to 454) was amplified by PCR, digested with NcoI/XhoI and cloned into pENTR4. The N-terminal Cdr2 fragment (residues 1 to 208) was cloned by PvuII/NcoI digestion of pENTR4-Cdr2. The inserts of the entry vectors were verified by DNA sequencing (Microsynth, Balgach, Switzerland).

*Cell culture and transient transfections*. Human HeLa cervical carcinoma, MCF-7 and BT474 breast carcinoma, SKOV3 and OVCAR3 ovary adenocarcinoma, HCT116 colorectal carcinoma, HepG2 hepatoma, U2-OS osteosarcoma, HEK293 embryonic kidney carcinoma, mouse embryonic fibroblasts lacking HIF-1$\alpha$ (MEFs Hif1a$^{-/-}$) or PHD1 (MEFs Phd1$^{-/-}$) cells were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM) (Sigma) as described previously (Camenisch et al., 1999). For long-term hypoxia, cells were grown in a gas-controlled glove box (Invivo$_2$ 400, Ruskinn Technologies, Leeds, UK). Transient transfections were performed using the polyethylenimine (Polysciences, Warrington, PA) method (Stiehl et al., 2006).

*Immunoblotting.* Immunoblot analyses were performed as previously described (Martin et al., 2005). Protein concentrations were determined by the Bradford method. Antibodies used were mouse monoclonal antibody (mAb) anti-HIF-1$\alpha$ (Transduction Laboratories, BD Biosciences), mAb anti-Cdr2 (Supplementary Figure S2), anti-mouse Cdr2 (Abcam), mAb anti-V5 (Invitrogen), mAb anti-myc (Roche Diagnostics, Rotkreuz,

Switzerland), mAb anti-β-actin (Sigma), rabbit polyclonal anti-PHD2 antibody (Novus, Abcam, Cambridge, United Kingdom), anti-mouse PHD2 (Novus), secondary polyclonal goat anti-mouse and anti-rabbit antibodies coupled to horseradish peroxidase (Pierce, Perbio, Lausanne, Switzerland). Chemiluminescence detection was performed using Supersignal West Dura (Pierce), and signals were recorded with a charge-coupled device camera (Lightimager LAS-4000 mini, Fujifilm, Bucher Biotec, Basel, Switzerland) or by exposure to X-ray film (Fujifilm, Dielsdorf, Switzerland).

*Co-immunoprecipitation.* HeLa cells were co-transfected with full-length or deletion constructs of pcDNA3.1/V5-Cdr2 and pcDNA3.1/myc-PHD1 and co-immunoprecipitation experiments were done as previously described (Barth et al., 2007).

*Reporter gene and mammalian one- and two-hybrid assays.* Cloning of the HIF-dependent firefly luciferase reporter gene constructs pGLTfHBSww and pGLTfHBSmm and pH3SVL was described previously (Rolfs et al., 1997; Wanner et al., 2000). Cells were co-transfected with 500 ng pH3SVL and 20 ng pRLSV40 Renilla luciferase reporter vector (Promega, Madison, WI, USA) to control for differences in transfection efficiency. Sixteen hours post-transfection, cells were equally distributed and exposed to 20% or 0.2% oxygen for another 16 hours. After washing with PBS and cell lysis with passive cell lysis buffer (Promega), luciferase reporter gene activity was determined using the dual-luciferase reporter assay system according to the manufacturer's instructions (Promega). Mammalian one- and two-hybrid analyses were performed using the mammalian Matchmaker system (Clontech, BD Biosciences) as previously described (Barth et al., 2007).

*mRNA quantification.* Total cellular RNA was extracted as described previously (Barth et al., 2007) and total tissue RNA was extracted using RNeasy Mini Kit (Qiagen, Hombrechtikon, Switzerland). First-strand cDNA synthesis was performed with 1-5 μg total RNA using reverse transcriptase (RT) and mRNA levels were measured by real-time quantitative (q) PCR using a SybrGreen qPCR reagent kit (Sigma) in combination with the MX3000P light cycler (Stratagene, Amsterdam, The Netherlands). Initial

template concentrations of each sample were calculated by comparison with serial dilutions of a calibrated standard. To verify RNA integrity and equal input levels, ribosomal protein L28 or S12 mRNA was determined, and the data were expressed as ratios relative to L28 or S12 levels.

*RNAi.* HeLa or MEF cells were transfected with 100 nM siRNA oligonucleotides using Lipofectamine 2000 (Invitrogen). The following stealth RNAi (Invitrogen) sequences were used:
control siRNA, forward 5'-gcuccggagaacuaccagaguauua-3';
control siRNA, reverse 5'-uaauacucugguaguucuccggagc-3';
hCdr2 siRNA#1, forward 5'-cucaacuccauucacaaauggaugc-3';
hCdr2 siRNA#1, reverse 5'-gcauccauuugugaauggaguugag-3';
hCdr2 siRNA#2, forward 5'-aacucuucauacuucaccuucaggg-3';
hCdr2 siRNA#2, reverse 5'-cccugaaggugaaguaugaagaguu-3';
hCdr2 siRNA#3, forward 5'-auauuccuccuccauagucacccgc-3';
hCdr2 siRNA#3, reverse 5'-gcgggugacuauggaggaggaauau-3';
mCdr2 siRNA #1, forward 5'-agguggagcuucuacggcaaaugaa-3';
mCdr2 siRNA #2, reverse 5'-uucauuugccguagaagcuccaccu-3';
mCdr2 siRNA #2, forward 5'-ccacucaaacgaagcagcagugaga-3';
mCdr2 siRNA #2, reverse 5'-ucucacugcugcuucguuugagugg-3';
hPHD2 siRNA, forward 5'-ggacgaaagccaugguugcuuguua-3';
hPHD2 siRNA, reverse 5'-uaacaagcaaccauggcuuucgucc-3';
mPHD2 siRNA, forward 5'-uccgucacguugauaacccaaaugg-3';
mPHD2 siRNA, reverse 5'-ccauuuggguuaucaacgugacgga-3'.

*Tissue specimen and TMA construction.* Two different TMAs were constructed as previously described (Kononen et al., 1998). A multitumor TMA containing 162 malignant and benign specimens from the following tissues: testis (12), placenta (4), breast (16), liver (12), lung (8), pancreas (8), ovary (8), colon (8), GIST (2), skin (6), brain (6), thyroid (8), uterus (8), kidney (16), bladder (8), prostate (12), tonsil (6), lymph node (8) and spleen (2). Twelve distinct cell lines from embryonal kidney (HEK-293-T),

melanoma (HA98, HN2004, PF2000), mesothelioma (MET5A), colon cancer (SW480), renal carcinoma (786-O), lung carcinoma (H69), breast carcinoma (MCF7, SK BR7), cervix (HeLa) and prostate cancer (PC3) were also included. In addition, a TMA comprising 384 RCC and normal kidney specimens collected from the University Hospital of Zürich (Zürich, Switzerland). All samples were histologically reviewed and selected for the study on the basis of hematoxylin and eosin-stained tissue sections. This study was approved by the local commission of ethics (ref. number StV 38-2005). Clinical information for the renal TMA was obtained for 331 cases. Tumors were graded according to the Thoenes grading system and histologically classified according to the World Health Organization classification (Eble et al., 2004).

*Immunohistochemistry.* TMA sections (2.5 μm) were transferred to glass slides followed by immunohistochemical analysis according to the Ventana automat protocols. The same antibody used for Western blot analysis was applied for detection of Cdr2 (1:20). Analysis was performed with a Leitz Aristoplan microscope (Leitz). Pictures of RCC specimens were taken with a digital camera (JVC, KY-070). The intensity of the staining was classified as follows: absent, weak, moderate, and strong staining.

*Statistical analysis.* Contingency table analysis, Chi-square tests, Kaplan-Meier curves, and log rank tests for evaluating correlations between Cdr2 and HIF-target genes as well as clinical parameters were calculated using StatView 5.0 (SAS, USA).

**Results**

*Onconeural antigen Cdr2 interacts with PHD1 but is oxygen-independently regulated*

PHD1 was expressed as fusion protein with the Gal4 DNA-binding domain (G4-DBD) or LexA as bait in two independent yeast two-hybrid screenings of mouse and human testis cDNA libraries. Two mouse Cdr2 clones (accession number NM_007672) and a human Cdr2 cDNA clone (accession number NM_001802) were identified. No interaction was observed between Cdr2 and PHD2 or PHD3 in yeast (data not shown). The Cdr2:PHD1 interaction was confirmed in a mammalian system by transient transfection of HeLa cells with V5-tagged Cdr2 and myc-tagged PHD1 followed by co-

immunoprecipitation (co-IP, Figure 1a). Additional co-IP experiments with Cdr2 deletion constructs demonstrated that PHD1 interacts with the N-terminal residues 1 to 208 of Cdr2 (data not shown).

To investigate whether the Cdr2:PHD1 interaction is oxygen-dependent, we expressed PHD1 fused to the G4-DBD together with the VP16 activation domain (VP16-AD) fused to Cdr2 in a mammalian two-hybrid system. As shown in Figure 1b, luciferase expression was significantly higher when the DBD-PHD1 and AD-Cdr2 fusion constructs were co-transfected than in transfections of either construct alone. In contrast to the significantly increased luciferase activity under hypoxic conditions following co-transfection of the positive control DBD-PHD1 with AD–HIF-1$\alpha$, luciferase expression remained unchanged after co-transfection of DBD-PHD1 with AD-Cdr2 under hypoxic conditions compared to normoxia. Whereas all three PHD isoforms interacted with HIF-1$\alpha$, strong luciferase expression was only observed when Cdr2 was co-transfected with PHD1. Normoxic luciferase actitivities of the positive controls are low, because AD-HIF-1$\alpha$ is constantly degraded.

PHD1 and Cdr2 were ubiquitously expressed in adult mouse tissues with most abundant mRNA levels in the testis (Supplementary Figure S1). To investigate whether Cdr2 gene expression is oxygen-dependently regulated by HIF-1, we analyzed Cdr2 mRNA levels in mouse embryonic fibroblasts (MEFs), either wild-type ($Hif1a^{+/+}$) or deficient ($Hif1a^{-/-}$) for HIF-1$\alpha$ (Figure 1c). Cdr2 mRNA levels were not regulated in a HIF-1$\alpha$-dependent manner under hypoxic conditions, while transcription of the known HIF target gene N-myc downstream regulated gene 1 (NDRG1) was induced. These results suggest that the Cdr2 gene is neither a HIF target nor is Cdr2 transcription oxygen-dependently regulated in MEF cells. In addition, no oxygen-dependent transcriptional regulation of Cdr2 was observed in cervical HeLa, ovary SKOV3 and OVCAR3 adenocarcinoma cells, breast BT474 and colorectal HCT116 carcinoma tumor cells (data not shown).

Even though Cdr2 mRNA is ubiquitously expressed, the only tissues reported to express Cdr2 protein are brain and testis as well as gynecological and breast tumors, indicating that Cdr2 is regulated at a post-transcriptional level (Corradi et al., 1997; Darnell et al., 2000). To investigate a possible Cdr2 protein regulation by PHD1, we

generated monoclonal antibodies against Cdr2 (Supplementary Figure S2). To this end, MBP-Cdr2 fusion protein was expressed in E. coli, affinity purified and used for the immunization of mice. Hybridoma supernatants were tested for the presence of anti-Cdr2 antibodies and one clone (mAb33), recognizing MBP-Cdr2 fusion protein as well as transfected Cdr2 by immunoblotting, was obtained (Supplementary Figure S2a). Neither mouse Cdr2 nor human Cdr3 was recognized by mAb33 (Supplementary Figure S2b). Importantly, mAb33 recognized endogenous Cdr2 in ovary SKOV3 cells and the specificity of the Cdr2 signal was confirmed by RNAi-mediated downregulation of Cdr2 gene expression (Supplementary Figure S2c). Anti-Cdr2 antibodies recognized two bands, running at ~62 and ~55 kDa, in cell lines as well as in *in vitro* transcription and translation experiments, suggesting that Cdr2 is post-translationally modified. Surprisingly, whereas the majority of ovary tumor tissues has been shown to react with PCD patient sera (Darnell et al., 2000), Cdr2 protein abundance in normoxic breast carcinoma cell lines MCF-7 and BT474 as well as in cervical HeLa, colorectal HCT116, hepatoma HepG2 and osteosarcoma U2-OS tumor cell lines was comparable to ovary adenocarcinoma SKOV3 and OVCAR3 cell lines (Supplementary Figure S2d).

To investigate whether $O_2$-dependent PHD activity is involved in the regulation of endogenous Cdr2 protein levels, we cultured ovary carcinoma SKOV3 cells under normoxic or hypoxic conditions, or in the presence of the PHD inhibitor dimethyloxalylglycine (DMOG). Although HIF-1$\alpha$ protein strongly accumulated in hypoxia and by PHD inhibition, endogenous Cdr2 protein abundance remained unaffected under these conditions (Figure 1d). We tested seven additional cell lines, (HeLa, OVCAR3, MCF-7, BT474, HCT116, HepG2 and U2-OS) as well as anoxia and anoxia/reoxygenation conditions, but found no evidence for PHD-dependent and/or $O_2$-dependent Cdr2 protein regulation (data not shown).

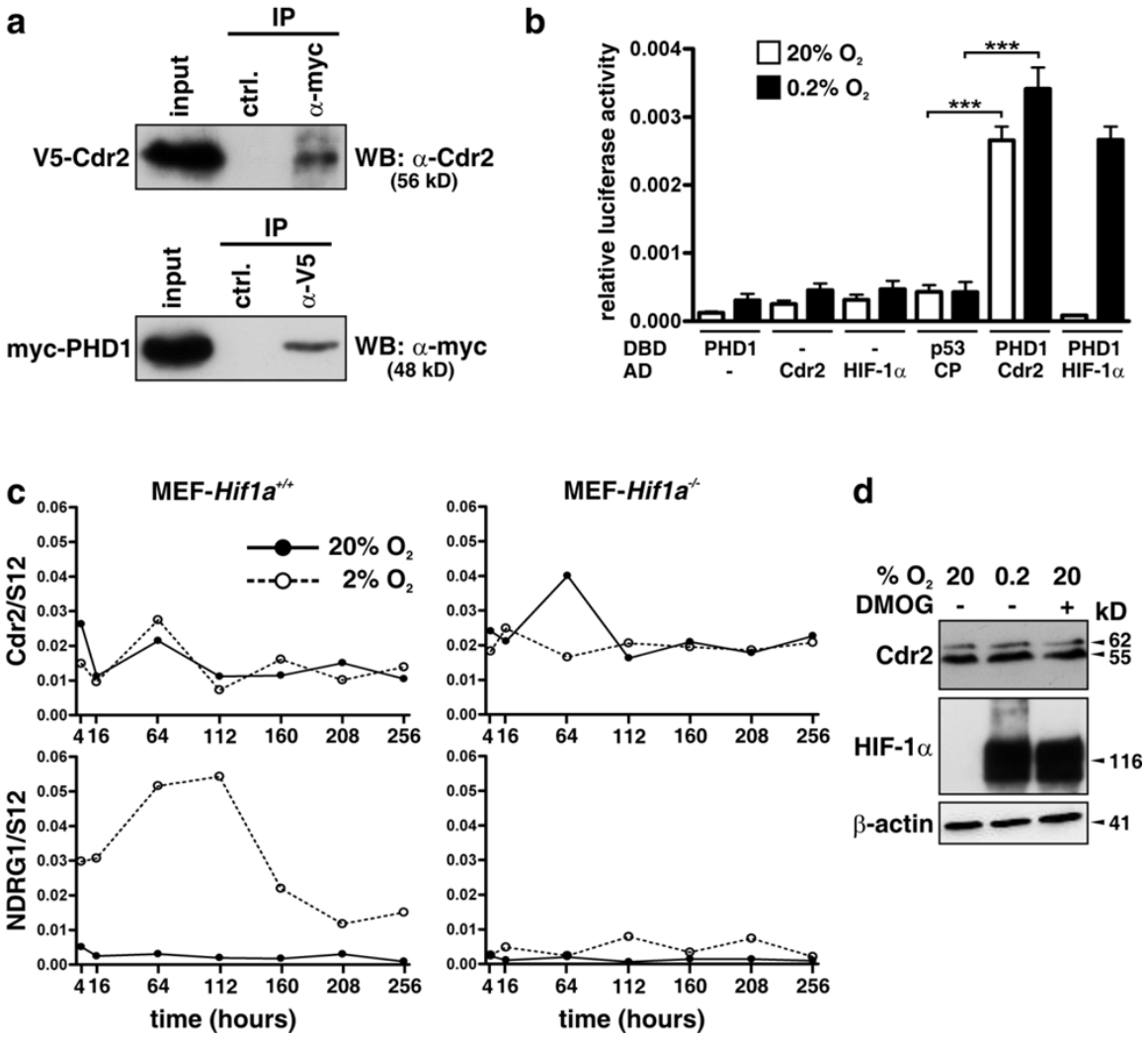



***Fig.1. Cdr2 interacts with PHD1 but is not regulated by oxygen***. ***(a)*** *Total cell extracts from HeLa cells transiently transfected with V5-Cdr2 and myc-PHD1 were incubated with anti-myc (upper panel) or anti-V5 (lower panel) antibodies or with isotype-matched control IgG, covalently bound to protein A agarose beads. Antibody-protein complexes were immunoprecipitated and analyzed by immunoblotting (IB) using anti-Cdr2 or anti-myc antibodies. IP, immunoprecipitate.* ***(b)*** *HeLa cells were transiently transfected with G4-DBD and VP16-AD fusion protein vectors and a Gal4-response element-driven firefly luciferase reporter, as well as a Renilla luciferase control vector. Following transfection, the cells were incubated under normoxic (20% $O_2$) or hypoxic (0.2% $O_2$) conditions, and luciferase reporter gene activities were determined 16 hours later. Mean values ± SEM of n=3 independent experiments are shown. Relative normoxic luciferase activities following transfection of G4-DBD-PHD1 were arbitrarily defined as 1.* ***(c)*** *$Hif1a^{+/+}$ and $Hif1a^{-/-}$ MEFs were cultured under normoxic (20% $O_2$) or hypoxic (2% $O_2$) conditions. Total RNA was extracted at indicated time points and mRNA levels of Cdr2, NDRG1 and ribosomal S12 were quantified by RT-qPCR. Transcript levels were normalized to S12 mRNA.* ***(d)*** *Ovary SKOV3 carcinoma cells were cultured under normoxic (20% $O_2$) or hypoxic (0.2% $O_2$) conditions, or in the presence of 1 mM DMOG for 16 hours. Whole cell extracts were analyzed by immunoblotting using anti-Cdr2 (mAb33), anti-HIF-1α and anti-β-actin antibodies.*

*Papillary renal cell carcinoma express high Cdr2 protein levels and show attenuated expression of HIF-target genes*

To analyze Cdr2 protein levels in human tumors, we performed immunohistochemistry analysis on 162 benign and malignant specimens of 20 different organs and 12 distinct cell lines using tissue microarray (TMA) technology (Kononen et al., 1998; Struckmann et al., 2008). As expected, Cdr2 staining was observed in brain as well as testis samples and somewhat weaker in breast and ovary tumors, but quite surprisingly the most intense Cdr2 protein signals were found in renal cell carcinoma (RCC) (data not shown). Subsequent analysis of a TMA containing 384 RCC and normal kidney samples revealed a significant difference ($P < 0.001$) in Cdr2 expression between histological RCC subtypes. Cdr2 protein expression was strongest in papillary RCC (pRCC) compared to clear cell (cc) and chromophobe RCC or benign oncocytoma (Table 1 and Figures 2a-c). No difference in Cdr2 protein levels was observed between papillary type 1 and type 2 RCC (data not shown). Due to the lack of high quality anti-PHD1 antibodies, we were not successful in analysing PHD1 protein expression in these human tumor samples. RT-qPCR analysis of renal cancer samples from the TMA showed that Cdr2 transcript levels were slightly reduced in pRCC compared to normal kidney, despite the very strong Cdr2 protein staining in pRCC (Figure 2d). Cdr2 mRNA levels were even more decreased in ccRCC (Figure 2e). Whereas PHD1 and PHD2 mRNA levels were slightly reduced in pRCC and ccRCC compared to normal kidney, induction of the HIF target genes PHD3, VEGF and CAIX by tumor hypoxia was strongly reduced in pRCC compared to ccRCC (Figures 2d and e). HIF-1$\alpha$and HIF-2$\alpha$ mRNA levels were comparable in pRCC and ccRCC. However, in contrast to ccRCC where over 50% of the tumor samples showed positive HIF-1$\alpha$ protein staining as expected, all pRCC were HIF-1$\alpha$ negative and decreased vascularization in pRCC compared to clear cell and chromophobe RCC or oncocytoma was evident by reduced CD34 staining (data not shown). Regulation of Cdr2 protein expression was VHL-independent (Supplementary Figure S3). In addition, normal kidney, pRCC and ccRCC tissues were analyzed for Cdr2 protein expression by immunoblotting (Figure 2f). Relative to normal kidney samples, Cdr2 protein levels were increased in pRCC and decreased in ccRCC, comparable to the TMA staining. Interestingly, additional faster

migrating bands were detected in pRCC samples and it remains to be investigated whether Cdr2 is alternatively spliced or proteolytically processed in pRCC.

| | % Cdr2 staining | | | |
|---|---|---|---|---|
| | **absent** | **weak** | **moderate** | **strong** |
| **chromophobe RCC** | **0 (0)** | **71.4 (10)** | **28.6 (4)** | **0 (0)** |
| **clear cell RCC** | **1.2 (3)** | **52.2 (133)** | **38.8 (99)** | **7.8 (20)** |
| **oncocytoma** | **0 (0)** | **71.4 (15)** | **28.6 (6)** | **0 (0)** |
| **papillary RCC** | **0 (0)** | **4.2 (2)** | **41.6 (20)** | **54.2 (26)** |

P value ($\chi^2$ test) for pRCC versus ccRCC < 0.001

***Table 1. Cdr2 immunostaining in chromophobe, clear cell and papillary RCC as well as oncocytoma***. *Absolute numbers of tumors are indicated in brackets.*

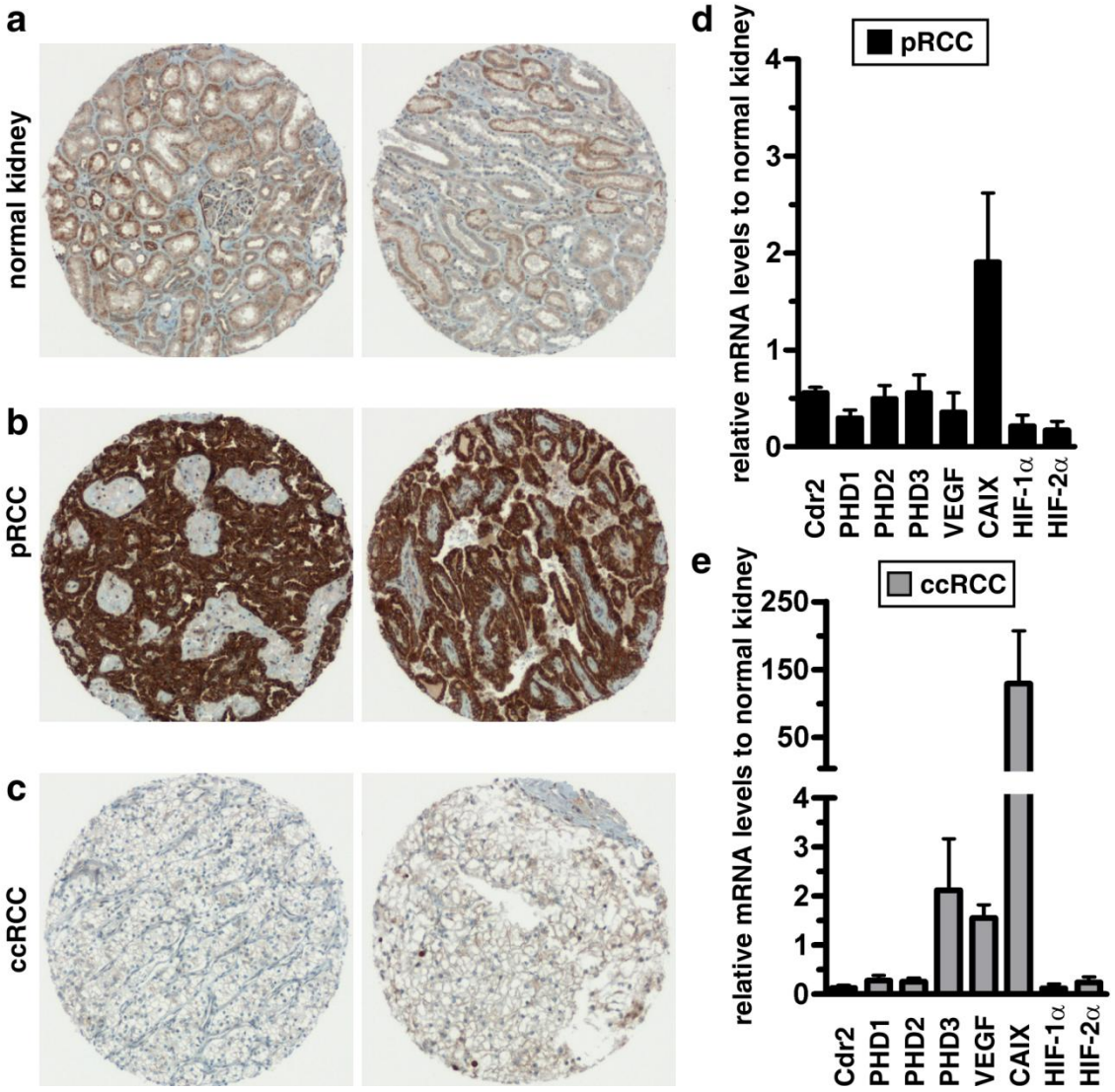



***Fig.2. Cdr2 protein levels are highly upregulated in pRCC. Examples from kidney*** *TMA immunostaining for Cdr2;* ***(a)*** *normal renal tissue.* ***(b)*** *papillary RCC.* ***(c)*** *clear cell RCC. Total RNA was extracted from 5 different normal kidney, papillary RCC* ***(d)*** *as well as ccRCC* ***(e)*** *samples and transcript levels of Cdr2, PHD1, PHD2, PHD3, VEGF, CAIX, HIF-1α and HIF-2α were quantified by RT-qPCR and normalized to ribosomal L28 mRNA levels. Normal renal tissue mRNA levels were arbitrarily defined as 1 and data presented as mean ± SEM relative to normal kidney transcript levels.* ***(f)*** *Whole cell extracts of TMA tissue samples were analyzed for Cdr2 (mAb33) and β-actin protein levels by immunoblotting.*

### *Cdr2 attenuates HIF-dependent transcriptional regulation*

Since neither Cdr2 gene expression nor protein abundance were regulated by HIF or PHD function, we next sought to investigate whether Cdr2 itself influences HIF-dependent gene regulation in tumor cells. Therefore, HeLa cells were transiently co-transfected with increasing amounts of Cdr2 or control lacZ expression vectors, together

with the HIF-dependent, HRE-driven reporter gene plasmid pH3SVL and a Renilla luciferase control plasmid. Notably, hypoxic luciferase activity was significantly reduced by Cdr2 overexpression compared to control transfections in a concentration-dependent manner (Figure 3a).

To determine whether attenuation of HIF-dependent gene activity by Cdr2 overexpression depends on the presence of functional HREs, we transiently co-transfected Cdr2 and HIF-dependent reporter gene constructs harboring either wild-type or mutated HREs from the transferrin enhancer. PHD2 overexpression was used as control. As shown in Figure 3b, significant suppression of hypoxic reporter gene activity by Cdr2 overexpression was dependent on functional HREs. That attenuating effects of Cdr2 are specific to HIF function could be confirmed by measuring HIF-dependent reporter gene activity in MEFs either deficient for HIF-1$\alpha$ (Hif1a$^{-/-}$) or transiently reconstituted with a HIF-1$\alpha$ expression plasmid (data not shown).

To analyze the role of Cdr2 in the transcriptional regulation of HIF-dependent genes in a HIF-1$\alpha$ wild-type background, total RNA was isolated from cells transiently transfected with lacZ, PHD2 or Cdr2 and cultured under normoxic or hypoxic conditions. Analysis of transcript levels of CAIX, VEGF, GLUT1 and PHD3 showed that expression of these genes was induced by hypoxia as expected but attenuated by Cdr2 overexpression (Figure 3c). These data suggest that exogenous Cdr2 overexpression leads to a suppressed hypoxic induction of HIF target genes. In contrast, transcript levels of GLUT1, CAIX, PHD2 and PHD3 were not regulated under hypoxic conditions or by overexpression of PHD2 or Cdr2 in Hif1a$^{-/-}$ MEFs (Supplementary Figure S4a). Notably, transient reconstitution of HIF-1$\alpha$ in these cells partially restored hypoxic induction of HIF-dependent reporter activity and the inhibitory effect mediated by Cdr2 overexpression (Supplementary Figure S4b). Quantification of HIF-1$\alpha$, HIF-2$\alpha$ and ARNT mRNA levels revealed no regulation by Cdr2 overexpression, indicating that attenuation of HIF-dependent transcriptional regulation is not at the mRNA level (Supplementary Figure S4c).

Furthermore, siRNA-mediated Cdr2 downregulation was used to evaluate whether modulation of endogenous Cdr2 gene expression affects HIF-dependent reporter gene activity. Compared to control siRNA transfection, HIF-dependent luciferase activity was

significantly increased by Cdr2 depletion (Figure 3d). PHD2 downregulation served as positive control and resulted in scarcely detectable normoxic HIF-1α protein levels. Immunoblot analysis was not sensitive enough to detect normoxic HIF-1α under Cdr2 RNA interference conditions. Neither PHD2 nor Cdr2 downregulation increased HIF-dependent luciferase activity under hypoxic conditions (data not shown).

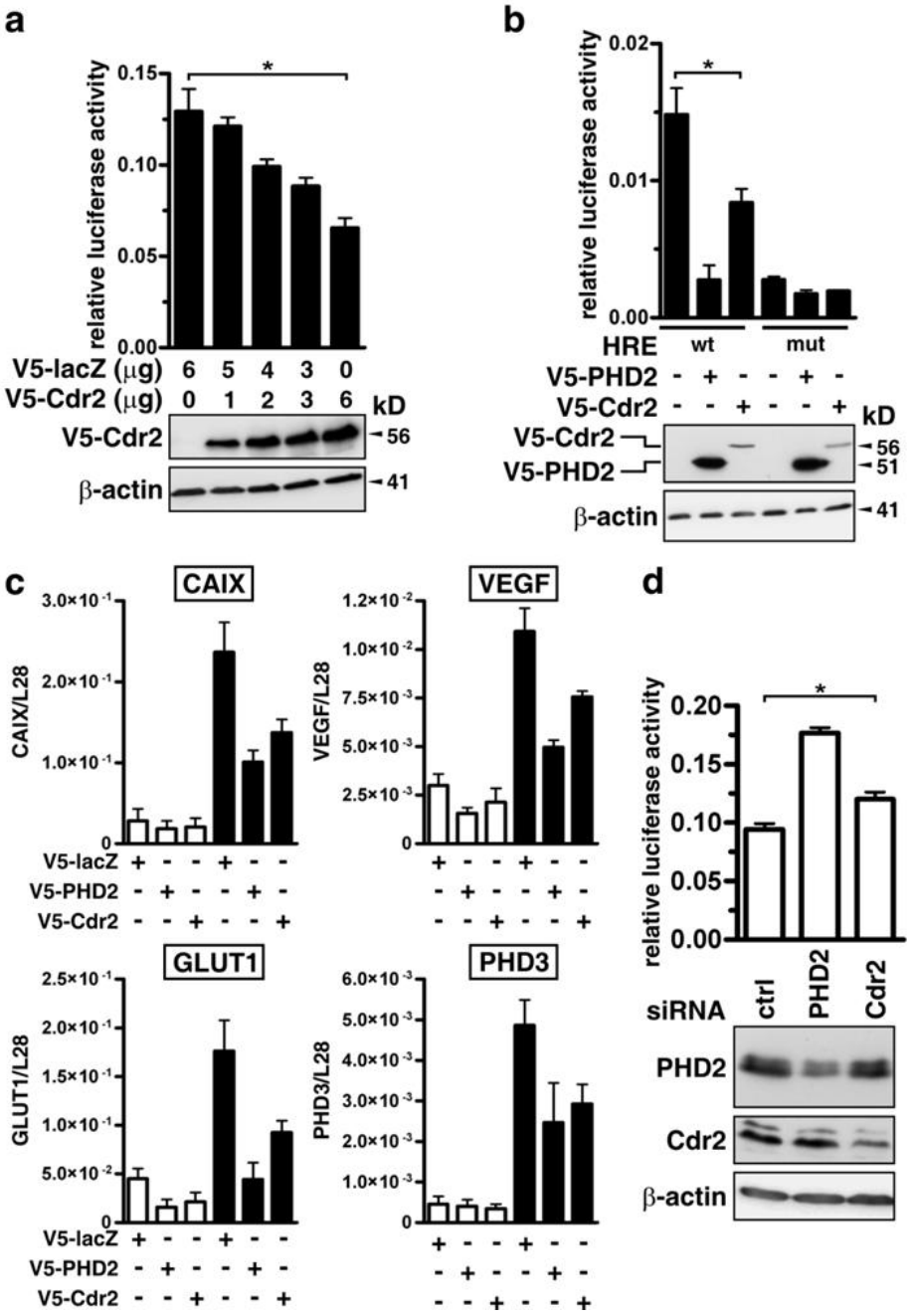


***Fig.3. Cdr2 attenuates HIF-dependent transcription**. **(a)** HeLa cells were transiently co-transfected with indicated amounts of Cdr2 or control lacZ expression vectors and pH3SVL as well as pRL-SV40 Renilla luciferase reporter vectors and cultivated for 16 hours under hypoxic (0.2% $O_2$) conditions before relative luciferase activities were determined. The results are mean values ± SEM of n=5 independent experiments performed in triplicates. **(b)** HeLa cells were co-transfected with Cdr2 or PHD2 expression vectors and wild-type or mutated HRE-driven luciferase reporter genes as well as pRL-SV40 and cultivated for 16 hours under hypoxic (0.2% $O_2$) conditions. **(c)** HeLa cells were transiently transfected with the indicated expression vectors and cultured under normoxic (open bars) or hypoxic (closed bars) conditions for 16 hours. Total RNA was extracted and CAIX, VEGF, GLUT1 and PHD3 transcript levels were quantified by RT-qPCR and normalized to ribosomal L28 mRNA. Data are mean values ± SEM of n=3 independent experiments. **(d)** HeLa cells were transfected with pH3SVL as well as pRL-SV40 and in addition with PHD2, control or Cdr2 siRNA oligonucleotides. Data are mean values ± SEM of n=3 independent experiments performed in triplicates. Endogenous Cdr2, PHD2, HIF-1α and β-actin protein levels were analyzed by immunoblotting. P values were obtained by unpaired t tests (**, $P < 0.01$).*

### *Cdr2 decreases hypoxic HIF-1α accumulation and modulates PHD1 protein levels*

We analyzed whether Cdr2 overexpression influences hypoxically stabilized HIF-1α protein levels. As shown in Figure 4a, overexpression of Cdr2 reduced hypoxic HIF-1α protein accumulation in a dose-dependent manner. To explore the concomitant role of Cdr2 and PHD1 in the negative regulation of HIF-1α stability and/or transactivation activity, we co-transfected pH3SVL and control pRL-SV40 reporter vectors together with Cdr2 and/or PHD1 expression plasmids. Hypoxic induction of HIF-dependent reporter gene activity was reduced by exogenous Cdr2 or PHD1 expression and an even stronger attenuation was observed when Cdr2 and PHD1 were co-overexpressed (Figure 4b). Strikingly, increased PHD1 protein levels were observed when Cdr2 was concomitantly transfected and immunoblot analysis of hypoxic endogenous HIF-1α protein accumulation under Cdr2 and PHD1 overexpression conditions showed that HIF-1α protein abundance was more efficiently reduced compared to Cdr2 or PHD1 expression alone (Figure 4b). Similarly to the human pRCC samples, endogenous PHD1 mRNA levels were not affected by Cdr2 overexpression suggesting that increased PHD1 protein levels result from a post-transcriptional mechanism (data not shown).

Next we investigated the effect of concomitant endogenous Cdr2 downregulation and myc-tagged PHD1 overexpression on HIF-dependent reporter gene activity (Figure. 4c). HIF-dependent luciferase activity was significantly augmented by Cdr2 siRNA, whereas it was reduced by V5-Cdr2 overexpression, confirming the results in Figure 3d

and 3a, respectively. In addition, Cdr2 siRNA-mediated elevated luciferase activity was significantly reduced by overexpression of increasing amounts of myc-PHD1 under normoxic as well as hypoxic conditions and repression of HIF-dependent luciferase activity under hypoxic conditions by myc-PHD1 expression was even more pronounced when V5-Cdr2 was co-transfected. Consistent with increased myc-PHD1 protein levels under Cdr2 overexpression conditions (Figure 4b), downregulation of endogenous Cdr2 prominently reduced myc-PHD1 protein abundance under normoxic and hypoxic conditions (compare lane 4 to 7 in Figure 4c), further suggesting a regulation of PHD1 protein abundance by Cdr2.

To investigate whether Cdr2-mediated suppression of hypoxic HIF activity is dependent on PHD1, we co-transfected pH3SVL reporter vector together with Cdr2 and/or PHD1 expression plasmids in PHD1-deficient ($Phd1^{-/-}$) MEFs. Indeed, whereas Cdr2 overexpression alone weakly increased HIF-dependent reporter gene activity, Cdr2 reduced relative luciferase activity when co-transfected together with PHD1, suggesting that the Cdr2-mediated effect was PHD1 dependent (Figure 4d). Vice versa, induction of HIF-dependent reporter gene activity by siRNA-mediated Cdr2 downregulation is also dependent on PHD1 (Figure 4e).

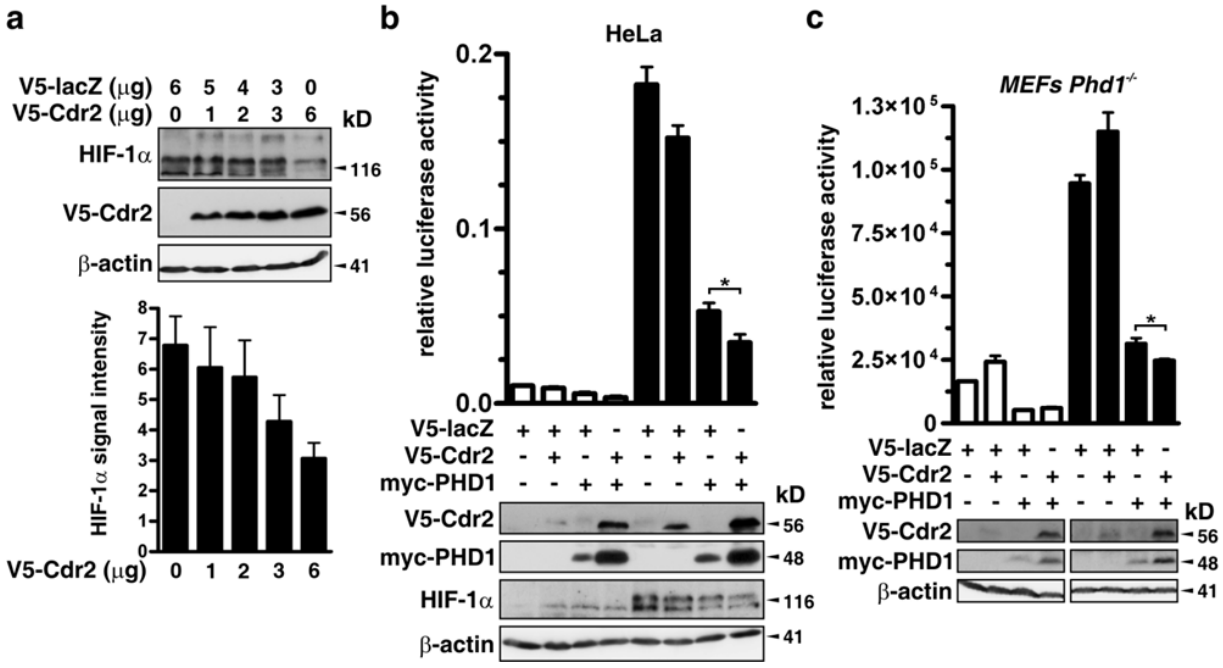



***Fig.4. Cdr2 overexpression reduces hypoxic HIF-1α protein accumulation. (a)*** *HeLa cells were transiently transfected with the indicated expression vectors and cultured under hypoxic conditions for 16*

*hours. HIF-1α, V5-Cdr2 and β-actin were detected by immunoblotting. A representative immunoblot is shown (top) and normalized hypoxic HIF-1α protein levels are presented as mean values ± SEM of n=3 independent experiments (bottom).* ***(b)*** *HeLa cells were transiently co-transfected with pH3SVL, pRL-SV40, lacZ, Cdr2 and/or PHD1 constructs and cultured under 20% $O_2$ or 0.2% $O_2$ conditions for 16 hours.* ***(c)*** *HeLa cells were transfected with pH3SVL, pRL-SV40 and V5-Cdr2 or myc-PHD1 as well as with Cdr2 or control siRNA oligonucleotides. Data are mean values ± SEM. Myc-PHD1, V5-Cdr2, Cdr2 and β-actin protein levels were analyzed by immunoblotting. P values were obtained by unpaired t tests (*, $P < 0.05$).* ***(d)*** *$Phd1^{-/-}$ MEF cells were transiently co-transfected with pH3SVL, lacZ, Cdr2 and/or PHD1 and cultured as in* ***b****. Luciferase activities were determined and normalized to total protein content in the lysates. Data are mean values ± SEM of n=3 independent experiments (**, $P < 0.05$).* ***(e)*** *$Phd1^{+/+}$ and $Phd1^{-/-}$ MEF cells were first transfected with Cdr2, PHD2 or control siRNA oligonucleotides and 24 hours later again with the same siRNA oligonucleotides together with pH3SVL and pRL-SV40. Data are mean values ± SEM of n=3 independent experiments performed in triplicates. Endogenous PHD2 and Cdr2 protein levels were analyzed by immunoblotting. P values were obtained by unpaired t tests (**, $P < 0.05$).*

### *Cdr2 represses HIF-1 transactivation activity by interfering with p300 recruitment*

We next examined whether Cdr2 might also interfere with HIF-1 transactivation activity. HIF-1α contains two transactivation domains (TAD) involved in the recruitment of general transcriptional co-activators, namely an N-terminal TAD (N-TAD) that overlaps with the oxygen-dependent degradation domain (ODDD) and a C-terminal TAD (C-TAD) (Figure 5a) (Jiang et al., 1997; Pugh et al., 1997). Using G4-DBD fusions with HIF-1α fragments containing either the N- or C-TAD in mammalian one-hybrid experiments, we observed that hypoxically induced C-TAD activity was significantly reduced by Cdr2 overexpression, whereas N-TAD activity remained unaffected (Figure 5b). Furthermore, HIF-1 C-TAD activity was reduced by Cdr2 in a concentration-dependent manner (Figure 5c).

To investigate whether recruitment of the general transcriptional co-activator p300 to the HIF-1α C-TAD is modulated by Cdr2, we performed mammalian two-hybrid experiments. HeLa cells were transiently co-transfected with G4-DBD p300 CH1 domain, VP16-AD HIF-1α CTAD and a Gal4-responsive luciferase reporter plasmid as well as Cdr2 or lacZ expression vectors. Incubation under hypoxic conditions resulted in a two-fold induction of luciferase activity which was significantly reduced by Cdr2 overexpression (Figure 5d). Proteasomal inhibition by MG132 has been shown to stabilize CITED2, which reduces HIF-1 transactivation activity by interfering with the HIF-1α:p300 interaction (Shin et al., 2008), and was used as positive control. Of note, we observed no significant interaction between Cdr2 and FIH under normoxic or

hypoxic conditions using mammalian two-hybrid analysis (data not shown). These results suggest that Cdr2 interferes with the recruitment of p300 by the HIF-1α C-TAD.

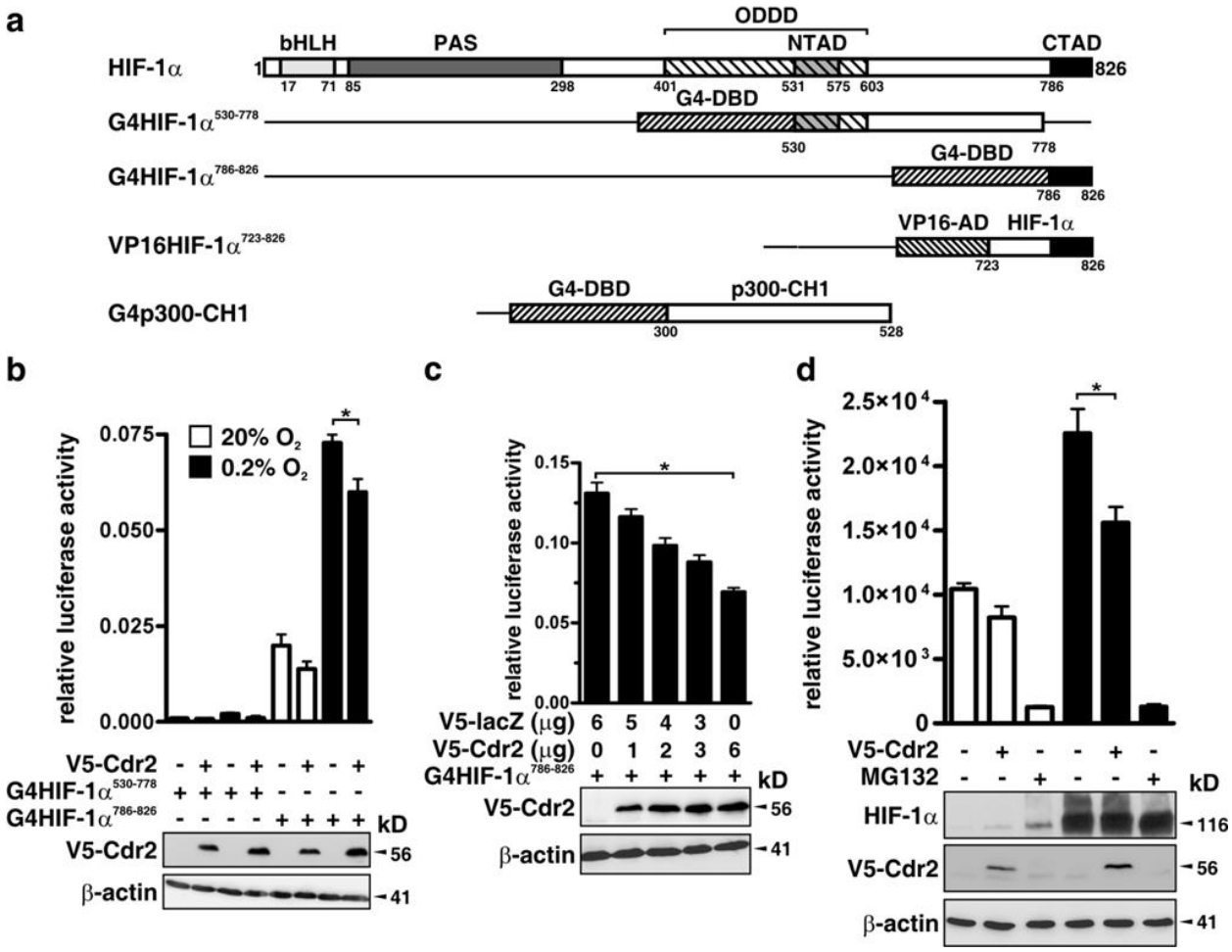



***Fig.5. Cdr2 reduces HIF-1 transactivation activity***. ***(a)*** *Schematic representation of the HIF-1α domain architecture and the G4-DBD and VP16-AD constructs used.* ***(b)*** *and* ***(c)****, HeLa cells were transiently co-transfected with pGH1α$^{530-778}$ or pGH1α$^{786-826}$ expression vectors* ***(b)*** *or pGH1α$^{786-826}$ alone* ***(c)****, the Gal4-response element driven firefly luciferase reporter pGRE5xE1b and a Renilla luciferase control vector, as well as with Cdr2 expression plasmids and lacZ control vectors* ***(c)****. Twenty-four hours post-transfection, the cells were cultured under either normoxic or hypoxic conditions for an additional 16 hours, and firefly luciferase activities were determined and normalized to Renilla luciferase activity. Expression of the transfected Cdr2 was verified by immunoblotting against V5 and β-actin served as loading control.* ***(d)*** *HeLa cells were transiently co-transfected with pG4CH1 (residues 300 to 523 of p300), pVP16H1α$^{723-826}$ and with Cdr2 expression plasmids as indicated. Twenty-four hours post-transfection, cells were cultured under normoxic or hypoxic conditions for 8 hours in the presence or absence of 5 μM MG132. Luciferase activities were determined and normalized to total protein content in the lysates. Hypoxic HIF-1α accumulation and overexpression of the transfected Cdr2 was verified by immunoblotting against HIF-1α, V5 and β-actin. Results are mean values of relative luciferase activities ± SEM of at least n=3 independent experiments. P values were obtained by unpaired t tests (*****, P < 0.05).*

**Discussion**

Cdr2 was identified in a search for novel PHD1 interacting proteins and comprehensive analysis of human tumor tissues revealed very strong Cdr2 protein expression in the papillary subtype of RCC. High Cdr2 protein levels correlated with strongly attenuated expression of HIF target genes in these solid tumors. Similarly, Cdr2 overexpression in different tumor cell lines reduced HIF-dependent transcriptional regulation, possibly due to both regulation of PHD1 protein expression and suppression of the transactivation activity of HIF-1$\alpha$.

Paraneoplastic neurological degenerations (PNDs) are a diverse group of human neurodegenerative diseases associated with cancer and represent probably the most evident examples of tumor immunity in humans (Albert and Darnell, 2004; Darnell and Posner, 2003a). It has been suggested that paraneoplastic cerebellar degeneration (PCD) is initiated when the neuron-specific protein Cdr2 is aberrantly expressed in breast and ovarian tumors and thereby recognized as foreign tumor antigen (Darnell and Posner, 2003b). PCD patients typically lack neoplastic symptoms and the disease only becomes evident when Cdr2-specific immune cells, mainly $CD8^+$ T cells, overcome immune tolerance in the brain and recognize neuronal Cdr2. Evidence for anti-tumor immune responses is supported by the fact that PCD patients show clinical and pathologic signs of suppression of tumor growth compared to other breast and ovarian cancer patients (Albert et al., 2000; Peterson et al., 1992). Furthermore, Santomasso et al. recently cloned the T cell receptor (TCR) genes from Cdr2-specific T lymphocytes. Subsequent expression in normal T cells transformed them into reactive cytotoxic T cells, able to lyse Cdr2-expressing human gynecologic tumor cells (Santomasso et al., 2007). Cdr2 can be considered as an important tumor antigen, since Cdr2 protein was detected in 25% of breast and 60% of ovarian tumors from patients who did not develop neurological disorders (Darnell et al., 2000), suggesting that neoplastic Cdr2 protein expression and development of Cdr2-specific immune cells might be independent of autoimmune responses. Therefore, a functional monoclonal anti-Cdr2 antibody might be of diagnostic importance for the detection of Cdr2 in human breast and ovarian tumors, and might enable the development of an onconeural antigen-specific TCR-based therapy.

Our findings indicate that Cdr2 represents an important tumor antigen also for pRCC, as 54.2% of these tumors demonstrated strong Cdr2 protein staining compared to 7.8% of ccRCC and none of chromophobe RCC or oncocytoma (Table 1). With 10% of all renal cell carcinomas, pRCC represents the most frequent RCC subtype after ccRCC (80%) (Kovacs et al., 1997; Motzer et al., 1996). Whereas deletion on chromosome 3 with the VHL gene residing on 3p25 has been clearly linked to ccRCC, genetic heterogeneity in pRCC is diverse, often involving chromosomal trisomies/tetrasomies in chromosome 7 and 17 as well as losses of chromosome Y (reviewed by Moch and Mihatsch, 2002). Cdr2 is localized on 16p12.3 and genetically not linked to pRCC.

Early signs of developing RCC are often absent and result in a high proportion of patients with metastases: 90-95% of these patients die within five years of diagnosis (Kosary and McLaughlin, 1993). Therefore, early detection of tumors is crucial and Cdr2 might become of diagnostic importance as novel pRCC tumor marker. In this regard, it will be important to determine whether anti-Cdr2 antibodies and/or Cdr2 protein are present in the serum of these patients. Interestingly, cellular host immunity has been suggested to be important in regulating tumor growth, because of late relapses after nephrectomy, sustained stabilization without systemic treatment and rare spontaneous regressions (Oliver et al., 1989; Vogelzang et al., 1992).

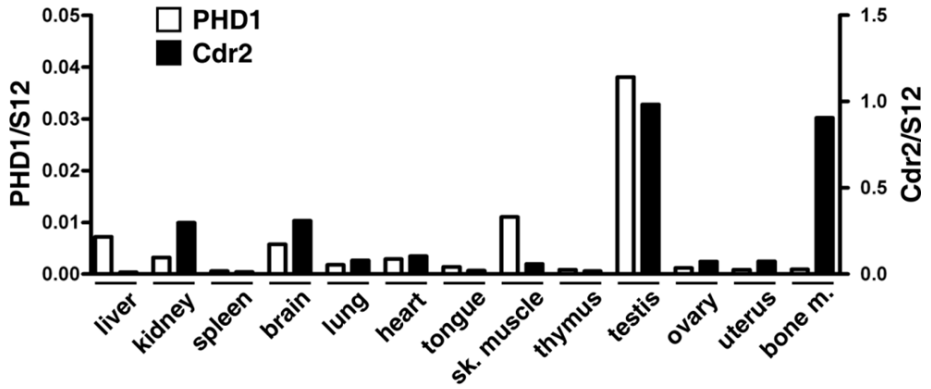
Only 7.8% of ccRCC showed strong Cdr2 staining and there was no correlation to an attenuated HIF response in theses tumors (data not shown). This might be due to the fact that 71% ccRCC were characterized by loss of VHL and a constitutive active HIF response pathway, leading to a proangiogenic state. In contrast, a recent examination of 791 RCC patients by angiography showed that pRCC were the most frequent hypovascular or avascular renal tumors (Onishi et al., 2002). We found no evidence for a non-functional HIF signaling system in pRCC but hypothesize that strong Cdr2 protein expression in these tumors, resulting in increased PHD1, decreased HIF-1$\alpha$ and hence suppressed activation of HIF target genes, might contribute to hypovascularization. Although pRCC is not a homogenous cancer, no difference in Cdr2 protein levels was observed between papillary type 1 and type 2 RCC and the

pathophysiological significance of Cdr2 protein expression in relation to the different pRCC types remains to be determined.

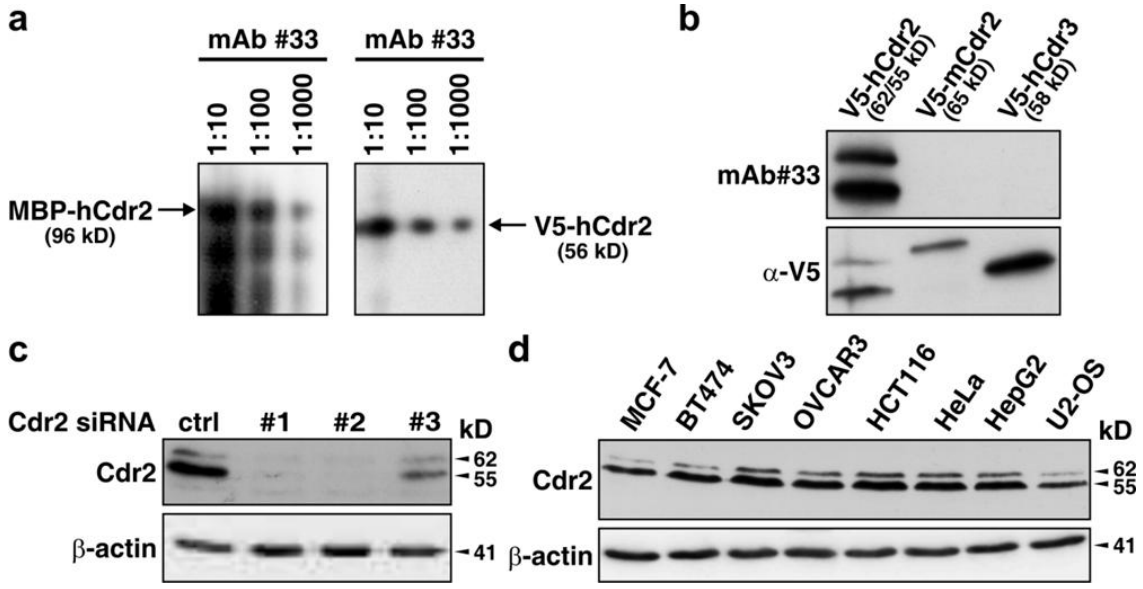
We showed that Cdr2 decreased HIF-dependent reporter as well as HIF target gene induction by suppressing both hypoxic HIF-1α protein accumulation and HIF transactivation activity. Vice versa, siRNA-mediated Cdr2 downregulation increased HIF-dependent reporter gene activity. Whereas PHD2 is generally considered to be mainly responsible for the normoxic turnover of HIF-α protein (Berra et al., 2003; Takeda et al., 2006), Erez et al. recently showed that overexpression of PHD1 in colon carcinoma cells inhibits tumor growth in nude mice (Erez et al., 2003). Increased PHD1 protein levels by Cdr2 overexpression might suggest that Cdr2 indirectly regulates HIF-1α protein abundance by elevating PHD1 levels.

Overexpression of Cdr2 also suppressed the transactivation activity of G4HIF-1α786-826, possibly by interfering with p300 recruitment. Oxygen-dependent HIF-1α asparaginyl residue 803 (N803) hydroxylation regulates HIF transcriptional activity by regulating p300/CBP recruitment. Cdr2 did not interact with FIH (data not shown) and it remains to be further investigated whether N803 is pivotal for HIF transactivation regulation by Cdr2. Interestingly, ectopic PHD2 expression in VHL-deficient cells has recently been shown to suppress HIF-1α transcriptional activity under hypoxic conditions without altering HIF-1α protein stability (To and Huang, 2005). Likewise, also the candidate tumor suppressor protein inhibitor of growth family member 4 (ING4) has recently been shown to associate with PHDs and affect HIF transactivation activity thereby regulating tumor growth and angiogenesis (Ozer et al., 2005).

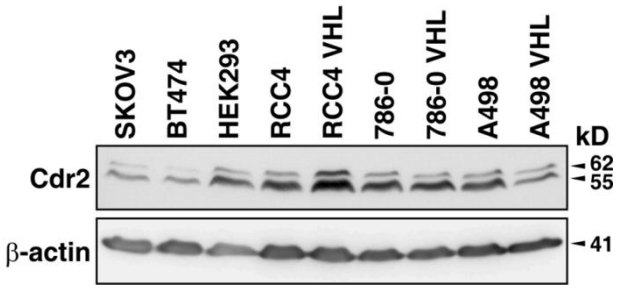
Taken together, we show that strong Cdr2 protein expression is specific for the papillary RCC subtype, leads to attenuation of the hypoxic response pathway, probably by regulation of PHD1 protein abundance as well as HIF transactivation activity, and propose Cdr2 as novel cancer tumor antigen for pRCC.



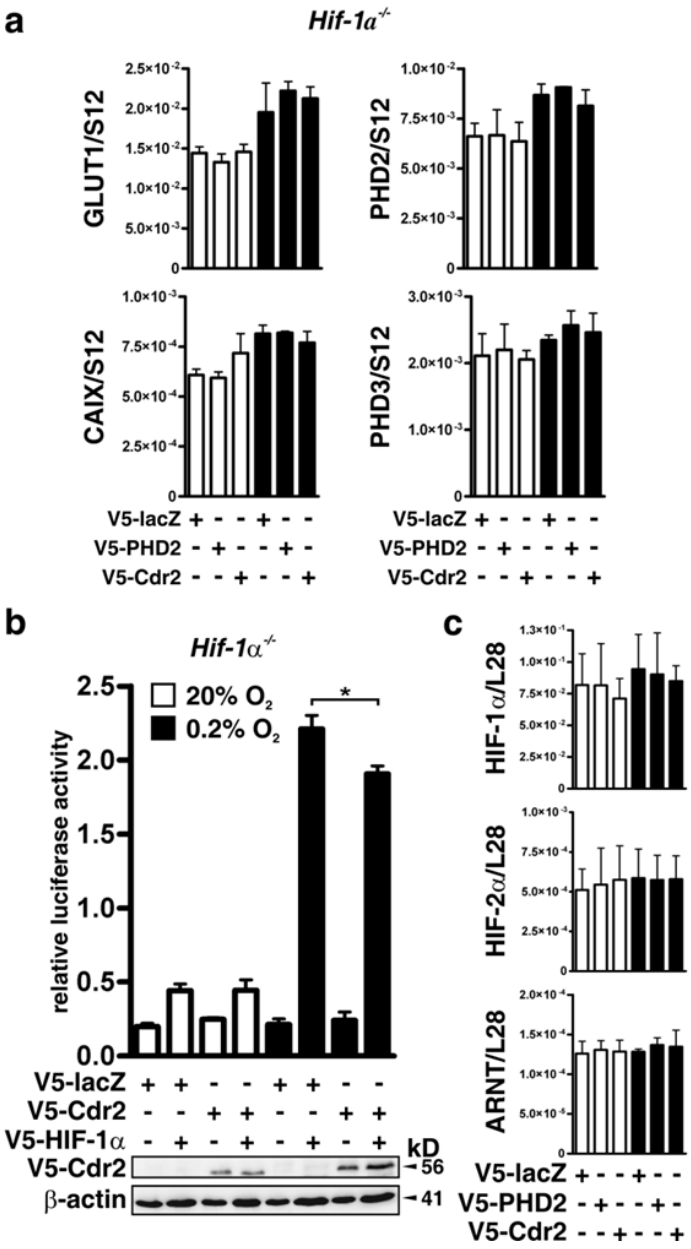

***Suppl. Fig.S1. Ubiquitous expression of Cdr2 and PHD1****. Total RNA was extracted from indicated adult mouse tissues and transcript levels of PHD1 and Cdr2 were quantified by RT-qPCR and normalized to ribosomal S12 mRNA levels. Note the different scales for Cdr2 and PHD1.*




***Suppl. Fig.S2. Generation of monoclonal anti-Cdr2 antibodies.*** *MBP-hCdr2 was expressed in E.coli (TB1) and expression was induced with 0.2 mM isopropyl thiogalactoside (IPTG) for 4 hours at 37°C before cell harvest. MBP-hCdr2 was purified using affinity chromatography on amylose beads and used for immunization of mice.* ***(a)*** *Hybridoma cell lines were established and culture supernatant from clone #33 tested against the antigen or lysates from V5-hCdr2 transfected HeLa cells by immunoblotting.* ***(b)*** *Monoclonal antibody mAb33 reacted specifically with human Cdr2 but not with in vitro transcribed and translated (IVTT) mouse Cdr2 or closely related human Cdr3. As control, the membrane was incubated with anti-V5 tag antibodies.* ***(c)*** *SKOV3 cells were transiently transfected with a control and three different Cdr2 siRNA oligonucleotides. Total cell lysates were prepared 24 hours post-transfection and Cdr2 as well as β-actin protein levels were determined by immunoblotting.* ***(d)*** *Indicated tumor cell lines were cultivated under normoxic conditions and total cell lysates analyzed by immunoblotting for Cdr2 as well as β-actin.*




***Suppl. Fig.S3. Cdr2 protein levels are VHL-independently regulated****. Ovary and breast carcinoma SKOV3 and BT474, embryonic kidney 293 as well as clear cell VHL-deficient as well as reconstituted RCC4, 786-0 and A498 cells were cultured under normoxic conditions and cellular extracts analyzed by immunoblotting for Cdr2 as well as β-actin.*




***Suppl. Fig.S4. Cdr2-dependent attenuation of the HIF response pathway requires functional HIF-1α.*** ***(a)*** *Indicated expression vectors were transiently transfected in Hif1a$^{-/-}$ MEFs and cells were cultured under normoxic or hypoxic conditions for 16 hours, before total RNA was extracted. Transcript levels of GLUT1, PHD2, CAIX and PHD3 were quantified by RT-qPCR and normalized to ribosomal S12 mRNA. Data are mean values ± SEM of n=3 independent experiments.* ***(b)*** *MEFs deficient for Hif1a were transiently transfected with pH3SVL as well as pRL-SV40 and the indicated expression plasmids. Twenty-four hours post-transfection, cells were cultivated under normoxic or hypoxic conditions for 16 hours before luciferase activity was measured. Results represent mean values ± SEM of n=3 independent experiments (*****, P < 0.05). Expression of transfected Cdr2 was verified by immunoblotting against V5 and β-actin.* ***(c)*** *HeLa cells were transiently transfected with lacZ, PHD2 or Cdr2 expression vectors and cultured under 20% $O_2$ or 0.2% $O_2$ conditions for 16 hours. Transcript levels of HIF-1α, HIF-2α and ARNT were quantified by RT-qPCR and normalized to ribosomal L28 mRNA. Data are mean values ± SEM of n=3 independent experiments.*

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## 3 Manuscript in Review

**Strong protein expression of the onconeuronal antigen Cdr2 correlates with high HIF prolyl-4-hydroxylase PHD1 levels in papillary renal cell carcinoma and is associated with a worse prognostic outcome**

Muriel R. Kaufmann[1], Peter Schraml[2], Thomas Hermanns[3], Roland H. Wenger*[1], and Gieri Camenisch[1]

[1]Institute of Physiology and Zürich Center for Integrative Human Physiology ZIHP, University of Zürich UZH, CH-8057 Zürich, Switzerland
[2]Department of Pathology, Institute of Surgical Pathology, University Hospital Zürich, CH-8091 Zürich, Switzerland
[3]Department of Urology, University Hospital Zürich, CH-8091 Zürich, Switzerland

*Correspondence should be addressed to Dr. Roland H. Wenger, Institute of Physiology, University of Zürich, Winterthurerstrasse 190, CH-8057 Zürich, Switzerland, Tel.: +41 (0)44 6355065; Fax: +41 (0)44 6356814; E-mail: roland.wenger@access.uzh.ch

**Keywords:** papillary renal cell carcinoma, paraneoplastic cerebellar degeneration, oxygen sensing, tumor marker

**Abstract**

Neoplastic expression of the onconeuronal cerebellar degeneration-related antigen Cdr2 in ovary and breast tumors is associated with paraneoplastic cerebellar degeneration (PCD). Cdr2 protein expression is normally restricted to neurons, but tumoral Cdr2 expression triggers tumor immunity, thereby constraining tumor growth. So far, aberrant Cdr2 expression has mainly been described for breast and ovarian tumors. Previously, we found strong Cdr2 protein expression in the papillary subtype of renal cell carcinoma (pRCC) and showed that Cdr2 interacts with the hypoxia-inducible factor (HIF) prolyl-4-hydroxylase PHD1. Conversely, high Cdr2 protein levels correlated with decreased HIF-dependent gene expression in cells as well as in clinical pRCC samples, providing a possible explanation why pRCCs are the most hypovascular renal tumors. Here, we demonstrate that strong Cdr2 protein expression in clinical samples from pRCC patients correlates with elevated PHD1 protein levels, suggesting that high PHD1 levels attenuate HIF-dependent gene expression. Interestingly, survival analysis revealed a negative correlation between Cdr2 expression and patient survival in clear cell (cc) RCC patients. These findings provide evidence that Cdr2 might represent an important tumor antigen in kidney cancer and possibly in other cancer types as well. In contrast to ovary and breast tumor patients that developed PCD, no Cdr2 auto-antibodies were detected in the serum of pRCC patients, which is in line with the fact that pRCC patients have not been reported to develop paraneoplastic neurodegenerative syndromes. This suggests that, despite a shared target antigen, tumor immunity and autoimmunity only partially overlap, and also highlights to which extent immuno-surveillance against cancer can be clinically silent.

**Introduction**

Paraneoplastic cerebellar degeneration (PCD) is often associated with lung and gynecologic tumors and is thought to develop as a consequence of an autoimmune reaction directed against Purkinje cells (Darnell and Posner 2006). It has been suggested that the neuron-specific cerebellar degeneration-related protein Cdr2 is expressed by breast and ovarian cancers and thereby elicits an anti-tumor response mediated by Cdr2-specific antibodies and T lymphocytes resulting in neurological symptoms (Albert and Darnell 2004; Darnell and Posner 2003). Although Cdr2 is an ubiquitously expressed gene, post-transcriptional regulation has been suggested to restrict Cdr2 protein expression physiologically to immune-privileged organs, such as brain and testis, and pathologically to ovarian cancers (Corradi *et al.* 1997; Darnell *et al.* 2000). However, Cdr2 protein levels were recently found to be comparably expressed in ovarian cancers and normal ovary tissue, as well as in tumors of other tissue origin. This suggests that Cdr2 antibody response in PCD might also be dependent on dysregulation of the immune system (Totland *et al.* 2011).

The physiological function of Cdr2 is incompletely understood. Cdr2 was found to interact with c-Myc in yeast and *in vitro* (Okano *et al.* 1999). Immunohistochemical analysis of rat brain sections showed a significant co-localization of Cdr2 and c-Myc in the cytoplasm of Purkinje neurons and overexpression of Cdr2 was shown to lead to a redistribution of c-Myc into the cytoplasm, where it co-localized with Cdr2 (Okano *et al.* 1999). More recently, Cdr2 was found to be regulated in a cell cycle dependent manner in cancer cells with highest protein levels during mitosis (O'Donovan *et al.* 2010).

Regions of low oxygen partial pressures (hypoxia) are found in many tumor types and contribute to malignant progression, therapy resistance and poor patient's prognosis (Brown and William 2004; Pouysségur *et al.* 2006). The master regulators of oxygen homeostasis are the heterodimeric hypoxia-inducible transcription factors (HIFs) and many of its target genes are involved in the adaptation of cancer cells to their hypoxic microenvironment (Wenger 2002; Wenger *et al.* 2005). Under normoxic conditions, a family of prolyl-4-hydroxylase domain (PHD) proteins hydroxylates the HIF$\alpha$ subunits, targeting them for proteasomal degradation (Bruick and McKnight 2001; Maxwell *et al.* 1999). In hypoxia, HIF$\alpha$ is stabilized, translocates to the nucleus and forms a powerful

transcription factor together with its counterpart HIF-1β/aryl hydrocarbon receptor nuclear translocator (ARNT) (Kaelin and Ratcliffe 2008; Schofield and Ratcliffe 2004).
We previously identified Cdr2 as a novel PHD1 interacting protein and found increased PHD1 protein levels when Cdr2 was concomitantly overexpressed in cells (Balamurugan *et al.* 2009). However, endogenous PHD1 protein expression in RCC patients awaited further analysis due to the lack of specific anti-PHD1 antibodies. In this study, we found high PHD1 protein levels in pRCC and a strong correlation between PHD1 and Cdr2 expression in tumor tissue microarray analysis. Despite the high Cdr2 levels, no Cdr2 auto-antibodies were detected in the sera of pRCC patients in contrast to patients diagnosed with PCD. This supports the notion that Cdr2 protein expression in cancer does not necessarily correlate with the development of paraneoplastic neurodegenerative disease and emphasizes the still poor mechanistic comprehension of these diseases.

**Materials and Methods**

*Immunoblotting.* Immunoblot analysis was performed as previously described (Balamurugan *et al.* 2009). Mouse anti-human Cdr2 was produced as described before (Balamurugan *et al.* 2009). Other antibodies used were rabbit anti-PHD1 (Novus Biologicals, Cambridge, United Kingdom, NBP1-40773) and mouse anti-β-actin (Sigma, Buchs, Switzerland). Secondary horseradish conjugated polyclonal goat anti-mouse and goat anti-rabbit antibodies were purchased from Pierce (Pierce, Lausanne, Switzerland). Chemiluminescence signals were detected using Supersignal West Dura substrate (Pierce) and signals were recorded with the LAS 4000 imaging system (Fuji, Bucher Biotec, Basel, Switzerland).

*ELISA*. 96-well ELISA plates (CANDOR Biosciences, Wangen, Germany) were coated with 50 µl recombinant Cdr2 capture antigen (200 ng/µl) (Balamurugan *et al.* 2009) diluted in Tris-Cl (pH 9.4) overnight at 4°C. Wells were washed three times with 200 µl PBS-0.05% Tween. 1% BSA in PBS was used to block the plate for 1.5 hours at room temperature. Horseraddish peroxidase conjugated goat anti human IgG F(ab)2-fragment from Dianova diluted 1/100,000 in 1/3 LowCrossbuffer (Candor Bioscience)

was used as the secondary antibody. All sera were diluted 1/400 in 1/3 LowCrossbuffer in PBS and analyzed in duplicates. The PCD serum samples were obtained from J. Honnorat. Ethical approval for the serum samples of RCC patients was obtained from the local ethics committee and Swissmedic (EK-1017 and EK-1634).

*Immunohistochemistry.* TMA sections (2.5 μm) were transferred to glass slides followed by immunohistochemical analysis according to the Ventana automat protocols. The same antibodies that were used for immunoblot analysis were applied for detection of Cdr2 (1:20) and PHD1 (1:10). Analysis was performed with a Leitz Aristoplan microscope (Leica Microsystems, Heerbrugg, Switzerland). Pictures of RCC specimens were taken with a digital camera (JVC, Reinach, Switzerland, KY-070). Since there were no negative cases for Cdr2, the weak and moderate cases were classified as 1 and the strong cases were classified as 2. Because general PHD1 staining was rather weak all positive cases were classified as 1 and the negative cases as 0.

*Kaplan-Meier survival analysis.* The data were compiled with the software package SPSS, version 15.0 (SPSS Software, München, Germany). Spearman's Rho was used to assess the statistical significance of the correlation between molecular and clinicopathological parameters. Univariate survival analysis was performed according to Kaplan-Meier (Kaplan and Meier 1958), and differences in survival curves were assessed with the log rank test. A p-value $< 0.05$ was considered statistically significant.

**Results**

*PHD1 expression strongly correlates with Cdr2 expression in pRCC*

To analyze PHD1 protein expression in clinical kidney samples that were previously probed for Cdr2 expression, immunohistochemical analysis (IHC) was performed on 384 RCC and normal kidney samples using tissue microarray (TMA) technology (Kononen *et al.* 1998; Struckmann *et al.* 2008). PHD1 expression was strong in pRCC, weak in ccRCC (Fig. 1a) and absent in chromophobe RCC or benign oncocytoma (data not shown). Furthermore, 74% of pRCC samples with high PHD1 protein levels showed strong Cdr2 staining and 67% of PHD1 negative samples showed weak Cdr2 staining (*p = 0.006). In addition, normal kidney, pRCC and ccRCC tissues were analyzed for PHD1 protein expression by immunoblotting (Fig. 1b). Interestingly, the expression of the smaller PHD1 isoform (40 kDa) was increased in pRCC and decreased in ccRCC samples compared to normal kidney. Quantification of PHD1 p40 and PHD1 p43 revealed that total PHD1 protein expression in pRCC was slightly higher compared to normal kidney and clearly higher compared to ccRCC (data not shown). As previously published Cdr2 protein expression was strongest in pRCC (Balamurugan *et al.* 2009). These findings suggest that Cdr2 might solely interact with the PHD1 p40 isoform.

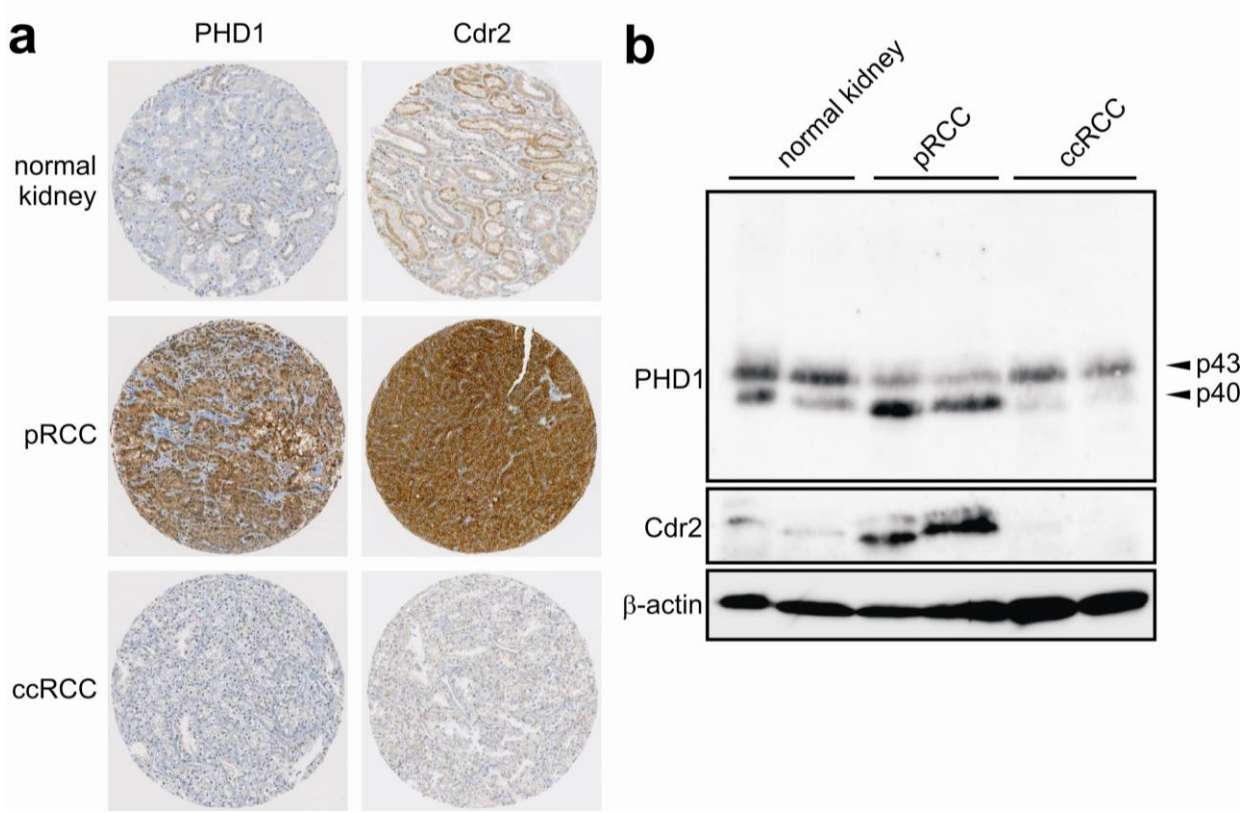


***Fig.1. PHD1 expression strongly correlates with Cdr2 expression in pRCC****. **a** Examples from kidney tissue microarray (TMA) immunostaining for PHD1 and Cdr2. **b** Whole cell extracts of TMA tissue samples were analyzed for PHD1, Cdr2 and β-actin protein levels by immunoblotting.*

Our findings indicate that Cdr2 represents an important tumor antigen not only for ovarian and breast cancer, but also for pRCC. To investigate whether high Cdr2 expression in pRCC also elicits an autoimmune response and leads to the generation of Cdr2 antibodies, an enzyme-linked immunosorbent assay (ELISA) using recombinant Cdr2 purified from bacteria was established. Cdr2-coated 96-well plates were incubated with sera from 5 PCD, 12 pRCC, 6 ccRCC and 5 non-cancerous patients to detect Cdr2 antibodies. No Cdr2 antibodies could be detected in any of the pRCC, ccRCC or control patients. In contrast, the sera of PCD patients showed high titers of Cdr2 antibodies and served as a positive control (Fig. 2a). These findings are in line with the fact that pRCC patients have not been described to develop symptoms of neurodegenerative disorders. Previously, Cdr2 has been reported to be increased in breast and ovarian cancer where it can cause an auto-immune response. Because we could demonstrate that Cdr2 is also strongly expressed in pRCC, we analyzed the correlation of Cdr2 expression with

patient survival (Fig. 2b). Cdr2 expression in RCC patients correlated with shorter patient survival times ($p < 0.005$).

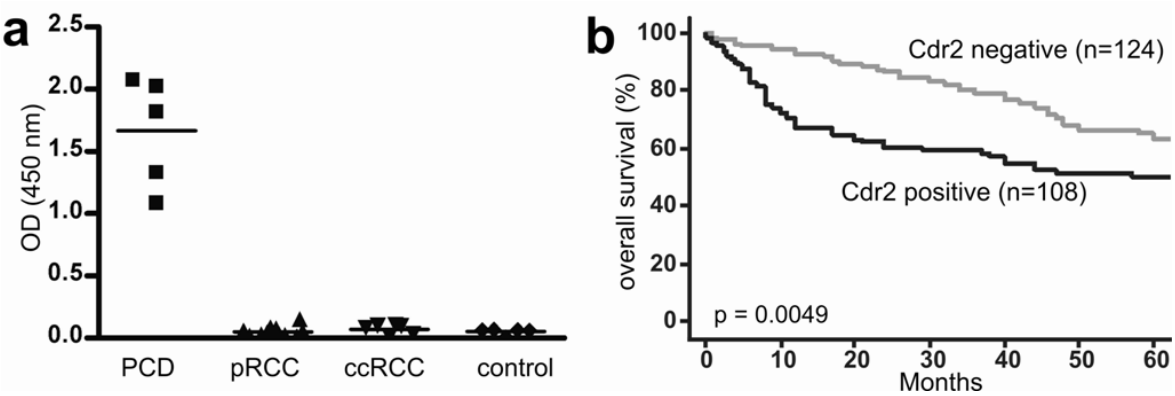


***Fig.2. Serum of RCC patients does not contain Cdr2 auto-antibodies. a*** *Serum samples from 5 PCD, 12 pRCC, 6 ccRCC and 5 non-cancerous patients were analyzed for Cdr2-auto-antibody abundance by ELISA.* ***b*** *Kaplan-Meier survival analysis of the relationship between the expression of Cdr2 and the length of patient survival was conducted in 232 ccRCC patients.*

## Discussion

We previously reported that Cdr2 is not only highly expressed in breast and ovarian tumors, but also in pRCC as 54.2% of these tumors showed strong Cdr2 protein staining. In contrast, only 7.8% of ccRCC and none of the chromophobe RCC or oncocytoma were found to express high levels of the Cdr2 antigen (Balamurugan *et al.* 2009). In addition, we found increased PHD1 protein levels when Cdr2 was overexpressed in HeLa cells, but were unable to analyze endogenous PHD1 levels in RCC samples because of the lack of specific anti-PHD1 antibodies (Balamurugan *et al.* 2009). Using a newly available anti-PHD1 antibody, PHD1 protein expression was analyzed in a TMA containing 384 RCC and normal kidney samples, in which we previously found Cdr2 to be highly expressed in pRCC (Balamurugan *et al.* 2009). We found that endogenous PHD1 protein expression is high in pRCC and correlates with strong Cdr2 expression. This indicates that high neoplastic Cdr2 protein levels might stabilize PHD1 protein expression, which in turn attenuates HIF-dependent gene expression.

Further analysis by immunoblotting revealed high expression levels of the PHD1 40 kDa isoform in pRCC compared to normal kidney. PHD1 has previously been shown to be

expressed as two different isoforms with molecular masses of 43 kDa and 40 kDa, respectively, due to the use of alternative translational initiation sites (Appelhoff *et al.* 2004). A subsequent study surveying further cell lines to examine the occurrence of these isoforms revealed that the preference of the isoform was largely cell type specific (Tian *et al.* 2006). The protein stability of the two isoforms varied greatly, p43 being the more stable isoform. Our finding that PHD1p40 but not PHD1p43 is highly expressed in pRCC suggests that Cdr2 might specifically regulate the protein stability of the smaller isoform. Since previous studies were based on overexpressed or endogenous PHD1 in HeLa cell culture, which express only one PHD1 isoform, the isoform specific regulation of PHD1 could not be investigated (Tian *et al.* 2006). Further experiments using A549 lung cancer, BT474 breast cancer or ND21 (a hybrid between mouse neuroblastoma and rat dorsal root ganglion neurons) cell lines could elucidate the Cdr2-specific regulation of the two PHD1 isoforms.

High titers of anti-Cdr2 auto-antibodies and Cdr2-specific CD8+ T cells are often found in the blood and CSF of PCD patients and are likely to contribute to the pathogenesis of PCD by provoking apoptosis and necrosis of Purkinje cells (Darnell and Posner 2003; Storstein and Vedeler 2007). However, a Cdr2-specific immune response does not necessarily lead to PCD since such auto-antibodies have been described in cancer patients without symptoms of PCD (Darnell and Posner 2003; Monstad *et al.* 2009; Monstad *et al.* 2006). Moreover, high Cdr2 expression was not only detected in ovarian tumors but also in other cancer types and in normal ovary tissue (Totland *et al.* 2011). It is therefore unclear whether Cdr2 auto-antibodies are mechanistically involved in the pathogenic progression of PCD or if they rather represent a marker for an aggressive neurological disease (Greenlee 2006). Since we found that Cdr2 is also highly expressed in pRCC, the abundance of Cdr2 auto-antibodies in pRCC patients was analyzed by ELISA. While Cdr2 antibodies could readily be detected in the sera of PCD patients, none of the tested pRCC patient harbored Cdr2 antibodies in the serum. This supports the notion that neoplastic Cdr2 protein expression does not always correlate with the generation of Cdr2 auto-antibodies and the development of PCD (Peterson *et al.* 1992). The molecular mechanisms of Cdr2-mediated neurological degeneration are poorly understood. Comprehensive attempts to establish animal models to investigate

the pathological progression of PCD have hitherto failed (Tanaka *et al.* 1995; Tanaka *et al.* 1994; Tanaka *et al.* 1995). Such models are urgently needed to better understand the mechanisms of the PCD development and to finally develop successful treatment strategies.

Hypoxia is a common feature of solid tumors (Endrich *et al.* 1979). It is strongly associated with malignant progression, metastatic outgrowth, resistance to therapy and overall poor prognosis in various tumor types (Brown 1998; Brown and William 2004; Pouysségur *et al.* 2006). Angiographic examination of 791 RCC patients revealed pRCCs to be the most frequent avascular or hypovascualar renal cancer subtype (Onishi *et al.* 2002). While there were not enough pRCC patients available to analyze survival rate, relatively high Cdr2 expression strongly correlated with decreased survival in ccRCC patients. This raises the possibility that a strong Cdr2 protein expression in tumors results in increased PHD1 expression and therefore decreased HIF-1$\alpha$expression. Hence, a suppressed HIF-dependent gene expression that might contribute to hypovascularization and therefore to a worse outcome. In line with this hypothesis, survival of ccRCC patients with high Cdr2 expression levels was significantly decreased compared to patients with low Cdr2 levels.

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## 4 Conclusion

Early signs of RCC are often absent and result in a high proportion of patients with metastasis. Early detection of tumors is therefore essential and Cdr2 might become of diagnostic importance as a novel pRCC tumor marker. We therefore tried to establish an indirect ELISA for the detection of Cdr2 antigen in the serum of pRCC patients. Due to the lack of a good second Cdr2 antibody, this aim has not been achieved yet. Following tumor markers are routinely tested in case of suspicion of a specific cancer (modified list from American Cancer Society)r

| Marker | Cancer type |
|---|---|
| Prostate-specific antigen (PSA) | Prostate cancer |
| Alpha feto-protein (AFP) | Liver cancer, testicular cancer |
| carcinoembryonic antigen (CEA) | Prognosis of colorectal cancer, not for diagnosis |
| CA125 | Treatment and post-treatment of ovarian cancer, not for screening |
| CA 72-4 | Ovarian and pancreatic cancer and cancers starting in the digestive tract, especially stomach cancer |
| Beta-2-microglobulin (B2M) | Multiple myeloma, chronic lymphocytic leukemia (CLL) and some lymphomas, also elevated in kidney disease and hepatitis |
| Bladder Tumor antigen (BTA) | In the urine of patients with bladder cancer |
| Chromogranin A | Neuroendocrine tumors such as carcinoid tumors, neuroblastoma and small cell lung cancer |
| Immunoglobulins | Antibodies, IgA, IgG, IgD and IgM, in bone marrow cancers such as multiple myeloma |
| Lactate dehydrogenase (LDH) | Testicular cancer and other germ cell tumors, not useful for diagnosis but for monitoring |
| Neuron-specific enolase (NSE) | Neuroendocrine tumors such as small cell lung cancer, neuroblastoma and carcinoid tumors, not for screening but for follow ups |
| S-100 | Melanoma |
| Thyroglobulin | Common forms of thyroid cancer |

***Table 1: Specific tumor markers suggested by the American Cancer Society***

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## Contributions to the Publications and Antibody Licensing

This thesis consists of the following original publications (in press or under review) and I contributed to it as indicated

**Muriel R. Kaufmann**, Sandra Barth, Bei Wu, Sascha Egger, Uwe Konietzko, Reiner Kunze, Hugo H. Marti, Meike Hick, Ulrike Müller, Gieri Camenisch, and Roland H. Wenger, *Dysregulation of hypoxia-inducible factor by presenilin/γ-secretase loss-of-function mutations.* The Journal of Neuroscience. (Revisions ongoing)

- Everything except Figure 1A

Balamurugan K, Luu VD, **Kaufmann MR**, Hofmann VS, Boysen G, Barth S, Bordoli MR, Stiehl DP, Moch H, Schraml P, Wenger RH, Camenisch G, *Onconeuronal cerebellar degeneration-related antigen, Cdr2, is strongly expressed in papillary renal cell carcinoma and leads to attenuated hypoxic response,* Oncogene, 2009; 28:3274-3285.

- Figure 1B, 4C – E

**Kaufmann MR**, Moch H, Wenger RH, Camenisch G, *Strong expression of the onconeuronal antigen Cdr2 correlates with high HIF prolyl-2-hydroxylase PHD1 levels in papillary renal cell carcinoma and is associated with a worse prognostic outcome;* submitted

- Everything except Figure 2A

Wrann S[#], **Kaufmann MR[#]**, Renato Wirthner, Stiehl D[§], Wenger RH[§], *HIF mediated and DNA damage independent phosphorylation of histone H2AX in chronic hypoxia; submitted*

[#,§] equal contributions

- Writing of the manuscript, manuscript attached in the appendix.

***Licensing of Cdr2 antibody***

Our mouse monoclonal antibody #33 against human Cdr2 (Balamurugan *et al.* 2009) has been licensed to Novus and is out for sale (NBP2-10509). My contributions to the licensing procedure:

- Testing and validating our hybridoma supernatant on different cell lines and by using different techniques (ELISA, IHC)
- Mycoplasma cleaning of hybridoma cell line and retesting the supernatant
- Retesting the nonfunctional antibody preparation of Novus by immunoblotting

# Appendix

## 1 Additional Manuscript

**HIF mediated and DNA damage independent histone H2AX phosphorylation in chronic hypoxia**

**Simon Wrann[#], Muriel R. Kaufmann[#], Renato Wirthner, Daniel P. Stiehl[§], and Roland H. Wenger[§]**

Institute of Physiology and Zürich Center for Integrative Human Physiology (ZIHP), University of Zürich, Winterthurerstrasse 190, CH-8057 Zürich, Switzerland

[#] equal contribution

[§] equal contribution

Correspondence: Dr. Roland H. Wenger, Institute of Physiology, University of Zürich, Winterthurerstrasse 190, CH-8057 Zürich, Switzerland, Tel.: +41 (0)44 6355065; Fax: +41 (0)44 6356814; E-mail: roland.wenger@access.uzh.ch

Running title: HIF-dependent H2AX phosphorylation

**Abstract**

The histone variant 2AX (H2AX) is phosphorylated at Ser139 by the PI3K-like kinase family members ATM, ATR and DNA-PK. Genotoxic stress is considered to be the main inducer of phosphorylated H2AX (γH2AX) which forms distinct foci at sites of DNA damage where DNA repair factors accumulate. γH2AX accumulation under severe hypoxic/anoxic (0.02% oxygen) conditions has recently been reported to follow replication fork stalling in the absence of detectable DNA damage. In this study, we found HIF dependent accumulation of γH2AX in several cancer cell lines and mouse embryonic fibroblasts exposed to physiologically relevant chronic hypoxia (0.2% oxygen) which did not induce detectable levels of DNA strand breaks. The hypoxic accumulation of γH2AX was delayed by RNAi mediated knockdown of HIF-1α or HIF-2α and further decreased when both HIF-αs were absent. Conversely, basal phosphorylation of H2AX was increased in cells with constitutively stabilized HIF-2α. These results suggest that both HIF-1 and HIF-2 are involved in hypoxic γH2AX accumulation which assumedly might increase the cell's capacity to repair potential DNA damage caused by reoxygenation.

**Keywords:** DNA damage response, oxygen sensing, tumor hypoxia

## Introduction

*Tumor hypoxia and therapy resistance*

Hypoxia is a common feature of solid tumors and develops due to inadequate vascularization, tortuous blood vessels and high oxygen consumption. Transient blockage of red blood cell flux alternating with rapid alleviation leads to frequent periodical hypoxia followed by reoxygenation (Yasui et al., 2010). Reoxygenation, most likely mediated by the generation of reactive oxygen species (ROS), but not hypoxia can lead to detectable DNA damage (Hammond et al., 2003). Hypoxia is strongly associated with malignant progression, metastatic outgrowth, genetic instability, resistance to radio-and chemotherapy and overall poor patient prognosis in various tumor types. (Brown, 1998; Brown et al., 2004; Pouysségur et al., 2006). Therefore, a thorough understanding of the molecular pathways in the hypoxic tumor microenvironment is warranted in order to develop new strategies for efficient cancer therapy.

Central to the cellular response to hypoxia is the heterodimeric hypoxia-inducible transcription factor HIF, consisting of one of three oxygen-labile $\alpha$ subunits and a common constitutive $\beta$ subunit (Wenger 2002; Schofield et al., 2004;). HIF activates a large number of oxygen regulated genes required for the adaptation of normal cells to hypoxia (Wenger et al., 2005). In tumors, HIF-1 is responsible for the generation of new blood vessels through transcriptional regulation of the vascular endothelial growth factor (VEGF), for pH regulation by increasing the expression of carbonic anhydrase (CA) IX, and for the aerobically increased glycolytic capacity of cancer cells, also known as Warburg effect (Seagroves et al., 2001; Minchenko et al., 2002; Svastova et al., 2004). Furthermore, hypoxic tumor cells are able to maintain metabolic functions without adequate oxygen supply via a switch to anaerobic fermentation (Pasteur effect) that is facilitated in a HIF-1 dependent manner (Schroeder et al., 2005). Therefore, high HIF-1 levels in the hypoxic tumor microenvironment is a well established factor for aggressive tumor growth and a negative factor for cancer therapy (Ryan et al., 1998; Ryan et al., 2000; Hopfl et al., 2002; Unruh et al., 2003; ).

Besides tumor hypoxia that leads to HIF-$\alpha$ protein stabilization, the loss of tumor suppressor proteins such as pVHL, p53 or PTEN or oncogenes such as v-src can

contribute to high HIF-α levels in cancer cells (Jiang et al., 1997; Krieg et al., 2000; Ravi et al., 2000; Zundel et al., 2000). Both HIF-1α and HIF-2α are widely overexpressed in many human cancers and are frequently associated with malignancy and poor prognosis (Birner et al., 2000; Aebersold et al., 2001). Furthermore, high HIF-1α protein levels have been shown to correlate with incomplete responses to chemotherapy and radiotherapy (Aebersold et al., 2001; Koukourakis et al., 2002; Bachtiary et al., 2003; Generali et al., 2006). Hypoxia *per se* affects radiation sensitivity since the radiation induced DNA damage is dependent on oxygen (Gray et al., 1953). In addition, decreased cell proliferation and lower drug concentrations in the hypoxic tumor areas contribute to the resistance to chemotherapy. However, the underlying molecular mechanisms causing therapy resistance of hypoxic tumor cells are incompletely understood, but it is likely that HIF downstream targets are directly involved in these processes.

*Targeting HIF to improve cancer therapy*

HIF-1 dependent hypoxic induction of the multidrug resistance MDR1 gene was one of the first described molecular mechanism explaining the involvement of HIF-1 in chemotherapy resistance in various tumor cells, including breast carcinoma, gastric cancer, colon cancer and glioma (Comerford et al., 2002; Wartenberg et al., 2003; Zhou et al., 2005; Nardinocchi et al., 2009). Hypoxically dysregulated apoptosis in response to chemotherapy might be another explanation (Erler et al., 2004; Sermeus et al., 2008). The role of HIF-1 in the regulation of apoptosis is very complex and context specific. The involvement of HIF-1 in apoptosis in certain cell types cannot be generalized since cells do not undergo apoptosis under degrees of hypoxia sufficient for HIF-1 induction (Wenger et al., 1998). In primary cells, hypoxia typically leads to cell cycle arrest and HIF-1 dependent apoptosis in case of more severe conditions (Greijer et al., 2004). However, HIF-1 functions as a robust suppressor of apoptosis in most transformed cells. We previously reported that transformed mouse embryonic fibroblasts (MEFs) were more sensitive to chemotherapy as well as to radiotherapy in the absence of HIF-1α due to an impaired DNA double-strand break (DSB) repair capacity (Wirthner et al., 2008). The underlying molecular mechanism involve markedly reduced expression of

DNA-PKcs, Ku80 and Ku70, three members of the DNA-dependent protein kinases (DNA-PK), in HIF-1α deficient MEFs. Our data were supported by a large number of studies that demonstrate reversal of radio- and chemoresistance by targeting HIF-1α in various tumor types (Zhang et al., 2004; Moeller et al., 2005; Williams et al., 2005; Brown et al., 2006; Li et al., 2006; Li et al., 2006; Song et al., 2006; Sasabe et al., 2007). For example, Li *et al*. showed that knockdown of HIF-1α in breast carcinoma cells repressed $G_0/G_1$ phase accumulation and relieved S phase block, thereby increasing sensitivity to chemotherapy and attenuating tumor growth (Li et al., 2006). Functional interference with HIF-1α in various tumor cells has been shown to result in enhanced cell death upon treatment with chemotherapeutic agents (Ricker et al., 2004; Peng et al., 2006; Hao et al., 2008; Sermeus et al., 2008; Flamant et al., 2010). On the other hand, experimentally increasing HIF-1α enhanced therapy resistance (Ji et al., 2006; Martinive et al., 2006). Of note, HIF-1 in germ cells of *Ceanorhabditis elegans* has recently been reported to antagonize p53-mediated apoptosis upon to DNA damage (Sendoel et al., 2010).

The induction of DNA damage by cytotoxic agents has proved to be an effective strategy for cancer therapy (Einhorn, 2002; Agarwal et al., 2003; Pires et al., 2012). Mutations in DNA damage response (DDR) genes can lead to increased frequency and incorrect DNA damage repair, thereby contributing to genomic instability characteristic for cancer cells (Bolderson et al., 2009). Because HIF-1 mediated therapy resistance was only observed when DSB but not single-strand break (SSB) inducing agents were applied, we suspected that HIF-1 might be involved specifically in DNA-DSB repair (Unruh et al., 2003).

*The DNA-damage response in hypoxia*

Upon DNA damage, histone H2AX is rapidly phosphorylated at Serine 139 by ataxia teleangiectasia mutated (ATM) kinase, ATM- and Rad3-related (ATR) kinase and DNA-PK (Fernandez-Capetillo et al., 2004; Zhang et al., 2006; Hurley et al., 2007). Previous studies suggested that severe hypoxia can elicit a DNA damage-like response, implying the activation of the ATR and ATM pathways and subsequent phosphorylation of H2AX (Hammond et al., 2003; Bencokova et al., 2009). More recently, Economopoulou *et al*.

identified a novel role for histone H2AX in hypoxia triggered angiogenesis (Economopoulou et al., 2009). Replication specific γH2AX was found to be induced in an ATR dependent manner in endothelial cells exposed to milder hypoxia (1% $O_2$). Whether HIF is involved in the hypoxic induction of γH2AX has not been analyzed so far. Therefore, we investigated a potential role for HIF-1 and HIF-2 in the phosphorylation of H2AX under chronically hypoxic (0.2% $O_2$) conditions. Hypoxic γH2AX induction was observed in a range of cancer cell lines, was delayed in HIF-1α deficient MEFs and after HIF-1α and HIF-2α knockdown in Hek293 cells, and was further decreased when both HIFs were downregulated. *Vice versa*, in 786-0 cells, devoid of pVHL and constitutively expressing HIF-2α, H2AX phosphorylation was increased, and could be reversed by pVHL reconstitution. These results suggest that HIF plays a crucial role in the DNA damage response under hypoxia.

**Materials and methods**

*Cell culture and lentiviral transductio.n* All cell lines were cultured in high glucose Dulbecco's modified Eagle's medium (DMEM; Sigma, Buchs, Switzerland) as described previously (Stiehl *et al.* 2006). For chronic hypoxic exposure, cells were grown in a gas-controlled glove box to handle the cells under constant oxygen (Invivo$_2$ 400, Ruskinn Technologies, Leeds, UK). Before medium change, all reagents were pre-equilibrated to the 0.2% oxygen containing gas mixture in the glove box. Cell number, size and viability were determined by trypan blue exclusion using an automatic cell analyzer (Vi-Cell, Beckman-Coulter, Nyon, Switzerland). Stable knockdown of HIF-1α and HIF-2α in HEK293 cells by RNA interference was achieved by lentiviral transduction of short hairpin (shRNA) constructs. Viral particles were produced in HEK293T human enbryonic kidney cells using the ViraPower lentiviral expression system according to the manufacturer's protocol (Invitrogen, Basel, Switzerland) as described previously (Stiehl *et al.* 2012).

*Immunoblot analysis.* Histone immunoblotting was performed as described previously (Wirthner *et al.* 2008). Primary antibodies used were: γH2AX (Millipore, Zug, Switzerland); total H2AX (Millipore), β-actin (Sigma, Buchs, Switzerland). Horseraddish

peroxidase coupled secondary anti-mouse and anti-rabbit antibodies were purchased from Pierce (Lausanne, Switzerland). Chemiluminescence detection was performed using Supersignal West Dura (Pierce) and signals were recorded and quantified using a charge-coupled device camera (Lightimager LAS-4000mini, Fujufilm, Dielsdorf, Switzerland). Extracted histones were stained with Ponceau S (Sigma).

*Flow cytometry.* Single cell suspensions were incubated with an antibody against γH2AX and propidium iodide (PI) according to manufacturer's instructions. Stained cells were analyzed with a FACSCanto II utilizing FACSDiva software (BD Biosiences, Allschwil, Switzerland).

*Single cell electrophoresis (comet assays).* Alkaline single cell electrophoresis was performed as described before (Wirthner *et al.* 2008). Briefly, MEFs were mixed with 0.5% low melting-point agarose (Sigma), solidified on microscopy slides, and lysed with 1% Triton-X100, 2.5 M NaCl, 100 mM EDTA, 10 mM Tris-HCl (pH 10.0) for 1 hour at 4°C in the dark. Horizontal electrophoresis (~0.74 V/cm; 300 mA) was performed in 300 mM NaOH, 1 mM EDTA for 30 minutes. Following SYBR green (Invitrogen) staining, DNA migration was visualized by fluorescence microscopy and the tail moment (% DNA in tail multiplied by tail length) was calculated from >150 cells per condition using the CometScore software package (TriTek, Sumerduck, VA, USA). Quantification of the median tail moments is shown as mean values ± standard error of the mean (SEM). Statistical analysis was performed applying two-tailed Student's *t*-test using GraphPad Prism version 4.0 (GraphPad Software, Ja Jolla, California, USA).

## Results

*γH2AX accumulation in chronic hypoxia*

Hammond *et al*. previously reported that severe hypoxia/anoxia (0.02% $O_2$) leads to ATR dependent γH2AX accumulation which was attributed to S-phase arrest (Hammond et al., 2002; Hammond et al., 2003). Because an atmospheric oxygen concentration of 0.02% $O_2$ results in a tissue partial pressure of oxygen which is most likely below the threshold for mitochondrial respiration, we investigated γH2AX induction under physiologically relevant hypoxic conditions. To ensure unimpaired mitochondrial respiration, an atmospheric oxygen concentration of 0.2% $O_2$ was chosen, corresponding to an oxygen partial pressure of approx. 1.5 mmHg. Reoxygenation induced ROS formation and DNA damage was prevented by replacing the cell culture medium every 24 hours with pre-equilibrated medium and by harvesting the cells inside of a hypoxic workstation. Several cancer cell lines were exposed to 0.2% $O_2$ for 3 to 72 hours, followed by analysis of H2AX Ser139 phosphorylation by immunoblotting. As shown in Figure 1A, γH2AX accumulated time-dependently in all six cell lines and reached maximal induction after 24 - 48 hours of hypoxic exposure, depending on the cell line. Hypoxic γH2AX induction in wild-type HEK293 cells with normal p53 was similar to SV40 large T antigen immortalized HEK293T cells, suggesting that p53 is not involved in hypoxic H2AX phosphorylation. Only wild-type HEK293 cells were used for subsequent experiments.

We next compared γH2AX accumulation in hypoxia with the effects of the topoisomerase II inhibitor and DSB inducing agent etoposide (Burden et al., 1998). γH2AX slowly accumulated in hypoxia with a maximum after 48 - 72 hours and declined after 96 hours (Figure 1B). One hour of etoposide treatment with concentrations from 0.25 - 8 μM resulted in a similar, dose-dependent increase in γH2AX levels. Total H2AX levels remained unaffected after both hypoxic exposure and etoposide treatment (Figure 1B).

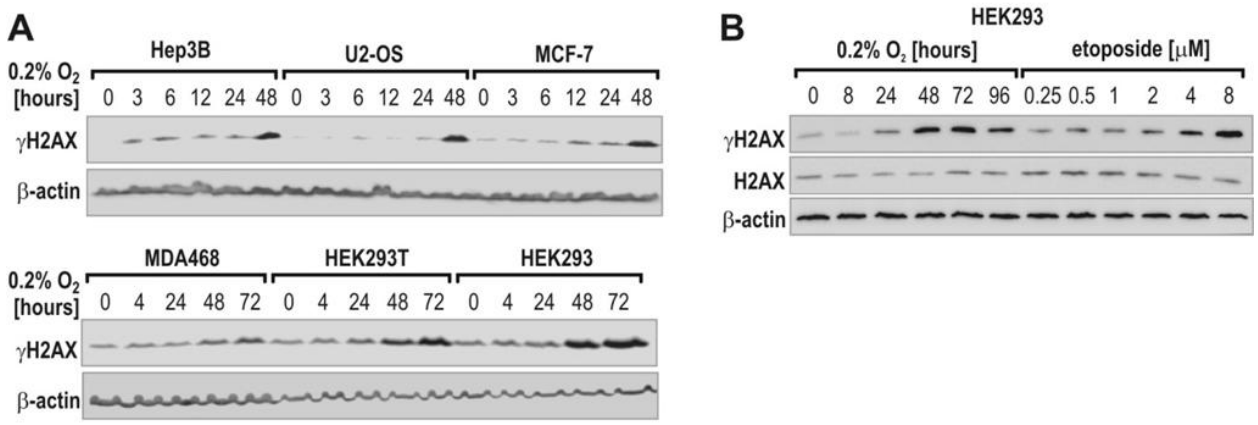


***Fig.1. Phosphorylation of H2AX in chronic hypoxia.*** *(**A**) The indicated cancer cell lines were cultured in 20% or 0.2% $O_2$ for up to 72 hours and γH2AX protein levels were analyzed by immunblotting. β-actin served as a control for equal loading and blotting. (**B**) HEK293 cells were exposed to 20% or 0.2% $O_2$ for up to 96 hours or to various etoposide concentrations up to 8 μM for one hour in normoxia. Phosphorylated and total H2AX were analyzed by immunoblotting.*

*Hypoxic γH2AX accumulation is HIF dependent*

The involvement of HIF in hypoxic H2AX phosphorylation was investigated by shRNA mediated stable knockdown of HIF-1α and/or HIF-2α in HEK293 cells. Hypoxic γH2AX accumulation was delayed after shRNA mediated knockdown of either HIF-1α or HIF-2α, with maximal levels only after 72 hours compared to 24 - 48 hours in the parental control (Figure 2A). Total H2AX remained unaffected (Figure 2A). Concomitant HIF-1α and HIF-2α double knockdown substantially decreased hypoxic phosphorylation of H2AX at all time points (Figure 2B).

To corroborate these findings, two different MEF cell lines derived from two different HIF-1α knockout mouse strains were analyzed. These cell lines were either only immortalized by SV40 large T (MEF-*Hif1a*$^{-/-}$T) or immortalized and transformed by H-*ras* (MEF-*Hif1a*$^{-/-}$*r*T), respectively (Feldser et al., 1999; Ryan et al., 2000). Importantly, these MEF cell lines were shown to lack functional HIF-2α protein (Park et al., 2003). Confirming the results obtained with HEK293 cells, γH2AX levels in wt MEFs accumulated after 24 hours exposure to 0.2% $O_2$, but were strongly impaired in MEFs devoid of HIF-1α. Total histone levels remained unaffected as shown by Ponceau S staining of the extracted histone fraction (Figure 2C). We previously reported increased susceptibility to DNA damage with enhanced phosphorylation of H2AX in MEF-*Hif1a*$^{-/-}$*r*T

upon low dose (0.5 - 4 μM) etoposide treatment (Wirthner et al., 2008). However, the HIF dependent difference of γH2AX levels decreased with higher doses of etoposide and was invisible upon treatment with 8 μM (Wirthner et al., 2008). In line with these findings, no HIF-1α dependent changes in γH2AX induction could be observed after high dose (8 μM) etoposide treatment which resulted in γH2AX levels that were only slightly higher than the γH2AX levels in HIF-1α positive MEFs after 24 hours of hypoxia (Figure 2C).

To further confirm the role of HIF in hypoxic γH2AX accumulation, parental and HIF-1α/HIF-2α double knockdown HEK293 cells were grown under 20% or 0.2% $O_2$ conditions for up to 72 hours before γH2AX levels were quantified by FACS analysis. While 88% of parental cells were strongly γH2AX positive after 48 and 72 hours of hypoxia, only 20 - 24% of the HIF-1α/HIF-2α double knockdown HEKs showed similarly elevated γH2AX staining (Figure 2D).

Finally, VHL-deficient 786-0 cells, containing constitutively stabilized HIF-2α (Maxwell et al., 1999), and reconstituted 786-0-pVHL cells were cultured under 20% or 0.2% $O_2$ conditions for 4 - 72 hours and analyzed by immunoblotting. In line with our findings above, both basal and hypoxic levels of γH2AX were substantially higher in 786-0 cells compared to 786-0-pVHL cells (Figure 2E).

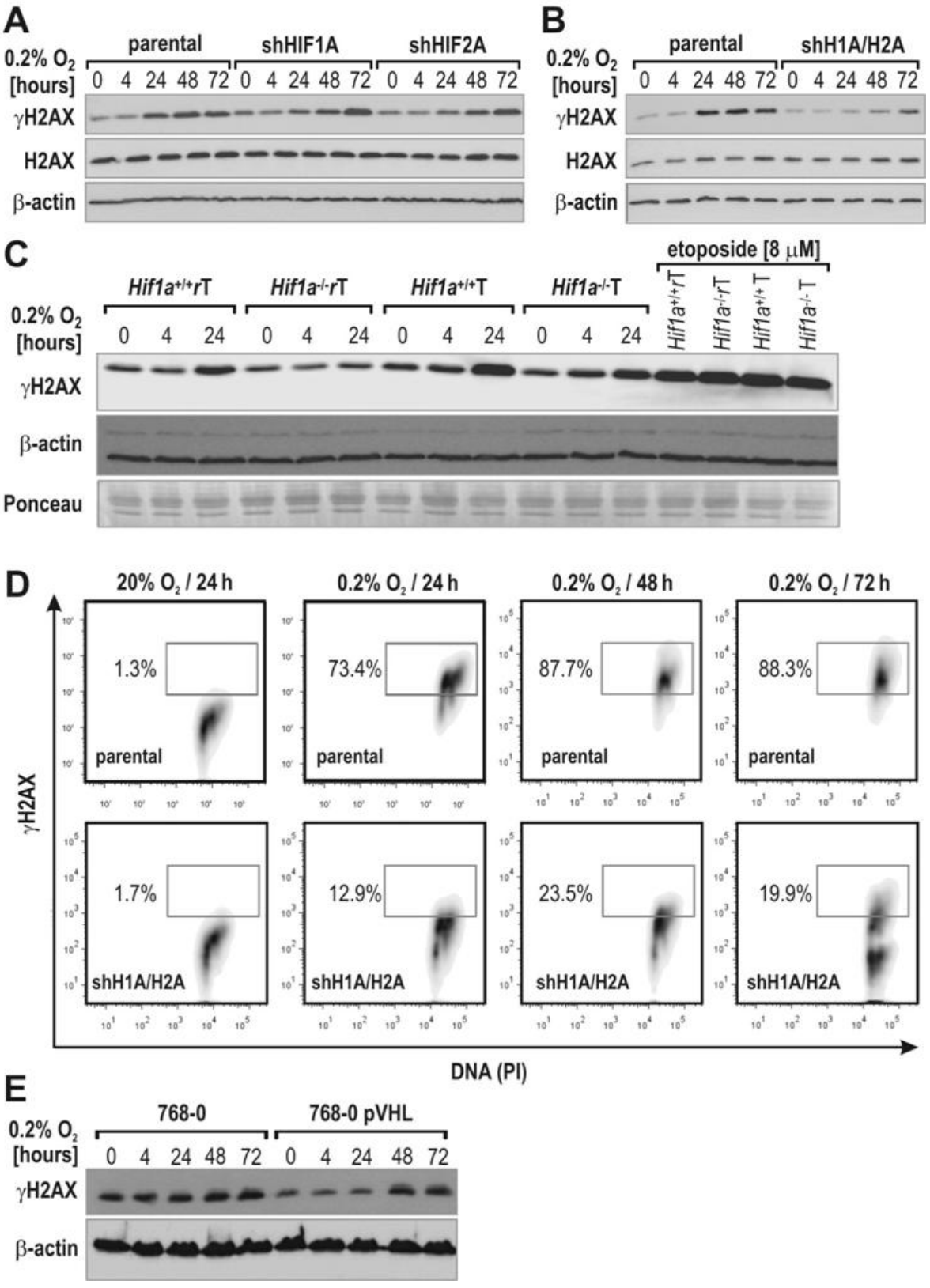


***Fig.2. HIFs are required for hypoxic γH2AX accumulation.*** *(**A, B**) Parental, shRNA-mediated HIF-1α or HIF-2α knockdown (shHIF1A or shHIF2A, respectively) or HIF-1α/HIF-2α double knockdown*

*(shH1A/H2A) HEK293 cells were grown under 20% or 0.2% $O_2$ conditions for the indicated time points. Phosphorylated and total H2AX was analyzed by immunblotting and β-actin served as a control for equal loading and blotting. (**C**) MEF-Hif1a$^{+/+}$rT, MEF-Hif1a$^{-/-}$rT, MEF-Hif1a$^{+/+}$T, MEF-Hif1a$^{-/-}$T were grown under 20% or 0.2% $O_2$ conditions for 4 or 24 hours or treated with 8 μM etoposide for 1 hour. Ponceau S staining was used as a control for equal extraction and loading of histones. (**D**) Parental and HIF-1α/HIF-2α double knockdown (shH1A/H2A) HEK293 cells were grown under 20% or 0.2% $O_2$ conditions for the time points indicated before γH2AX levels were analyzed by FACS. γH2AX positive cells were gated as indicated by the rectangles and quantified relative to the total cell number. (**E**) 768-0 and 786-0-pVHL cells were grown under 20% or 0.2 % $O_2$ conditions for 24 to 72 hours and γH2AX and β-actin protein levels were analyzed by immunoblotting.*

### *Hypoxic γH2AX accumulation is independent of DNA-DSB formation*

Hypoxia has previously been suggested to induce genetic instability, associated with increased HIF-1α levels (Bristow et al., 2008). However, the previously published lack of detectable DNA damage at 0.02% $O_2$ suggests that hypoxic γH2AX accumulation might be partially or fully independent of DNA-DSB formation (Hammond et al., 2003). To directly assess DNA-SSB and DNA-DSB formation under 0.2% $O_2$ conditions, we performed alkaline single-cell electrophoresis (comet assays) in wild-type and HIF-1α deficient MEFs and concomitantly determined γH2AX protein levels by immunoblotting. As shown in Figure 3A, emergence of DNA-DSB induced by 1 μM etoposide could be visualized reliably by "comet halo" formation. Quantification of the median of the tail moment demonstrated a significant ($p < 0.0001$) 4-fold increase following treatment with 1 μM etoposide for 1 hour but not after up to 24 hours of 0.2% $O_2$ (Figure 3B, upper panel). In contrast, γH2AX levels in HIF-1α wild-type MEFs were even higher after 12 and 24 hours of hypoxia than following treatment with 1 μM etoposide (Figure 3B, lower panel). Taken together, these data suggest that DNA-DSB is not a major determinant of hypoxic γH2AX induction.

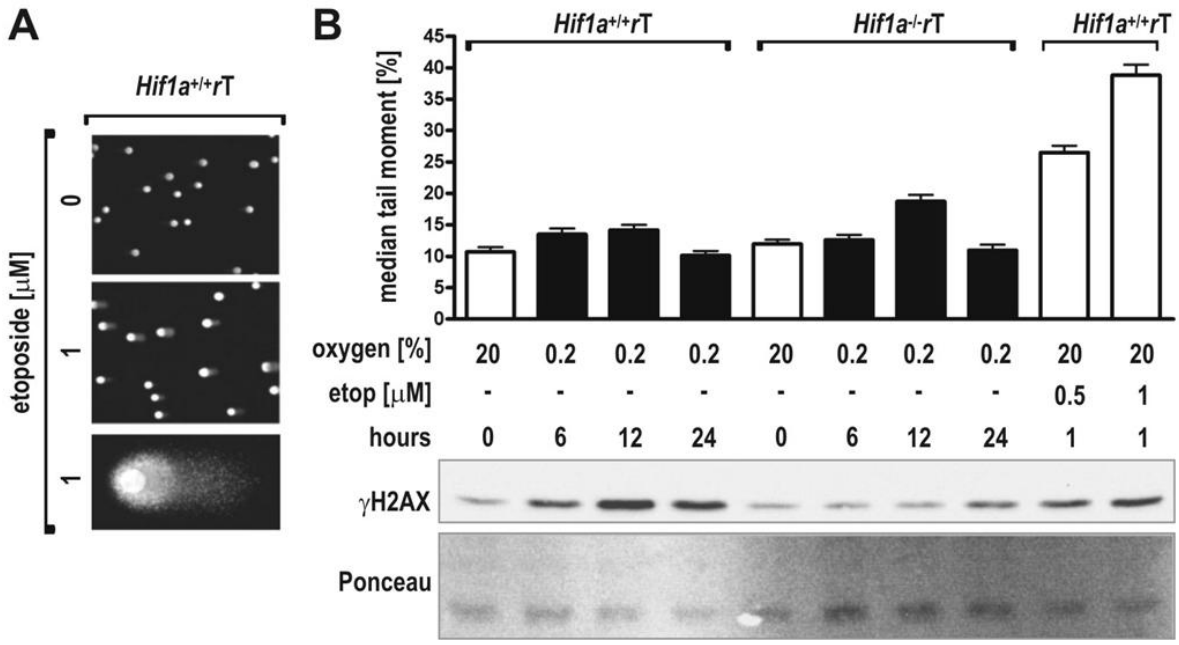


***Fig.3. Hypoxia does not induce detectable DNA strand breaks***. *(**A**) Representative example of a comet assay. DNA fragmentation in wildtype MEFs was induced by exposure to 1 μM etoposide for 1 hour. DNA was stained with SYBR green and all images were acquired with fixed exposure times. (**B**) DNA fragmentation was quantified by determining the median tail moment of at least 150 comets per condition using CometScore software. Data are shown was mean values ± SEM.*

**Discussion**

Hypoxic regions in solid tumors result from an imbalance between cellular oxygen consumption and oxygen delivery as a consequence of inefficient tumor vasculature and limited oxygen diffusion (Chitneni et al., 2011). Rapid and frequent variations in red blood cell flux cause temporal and spatial variations in the degree of hypoxia within the same tumor. We found that chronic hypoxia triggers the phosphorylation of the histone variant 2AX in a HIF dependent manner. In line with a previous report (Hammond et al., 2003), we showed that γH2AX levels after chronic hypoxia were comparable with etoposide treatment. Hypoxia (0.2% $O_2$) did not lead to detectable DNA damage when analyzed by alkaline single cell electrophoresis. Furthermore, proliferation and cell viability were not altered, even after long term (3 days) hypoxic exposure (data not shown). However, conditions close to anoxia have been reported to have direct cytotoxic effects and elicit apoptosis (Papandreou et al., 2005). In line with our previous findings (Wirthner et al., 2008), 53BP1 dose-dependently accumulated in distinct nuclear foci upon treatment with etoposide and partially overlapped with γH2AX staining (data not shown). These foci are most likely sites of DNA-DSBs. In contrast, in chronic hypoxia γH2AX did not accumulate in nuclear foci but showed a more diffuse pattern throughout the nucleus (data not shown). A similar granular γH2AX and ATM phospho-S1981 staining has been reported previously to occur in response to severe hypoxia (0.02% $O_2$) (Hammond et al., 2003; Bencokova et al., 2009). Hammond *et al*. found that severe hypoxia leads to replication fork stalling and ATR dependent γH2AX accumulation during S-phase (Hammond et al., 2002; Hammond et al., 2003). Moreover, diffuse and pan-nuclear γH2AX staining has been found to occur upon non-ionizing UV-C irradiation, independent of DNA-DSBs (Marti et al., 2006). Infection with inactivated adeno-associated virus has been shown to lead to replication fork stalling and a diffuse γH2AX nuclear staining which was essential for subsequent cell cycle arrest in the absence of DNA damage (Fragkos et al., 2009). However, the mechanism behind this diffuse γH2AX distribution pattern as well as its functional relevance are currently unknown.

DNA-DSBs are serious lesions that can lead to genomic instability if improperly repaired, or ultimately to cell death if the repair machinery is saturated. It is essential

that the cell closely monitors such stress conditions and initiates signals for an adequate response. Phosphorylation of H2AX on serine 139 is established as a sensitive marker for DNA-DSBs (Bonner et al., 2008). γH2AX is regarded as a key component for DNA repair, even though it seems dispensable for the initial recognition of DNA-DSBs and H2AX-deficient mice are viable (Celeste et al., 2002; Celeste et al., 2003).

The physiologic relevance of hypoxia induced γH2AX is poorly understood. A recent report showed that hypoxia triggered neovascularization required endothelial H2AX and γH2AX was induced in an ATR dependent manner in moderate hypoxia due to replicative stress (Economopoulou et al., 2009). Genetic inactivation of H2AX was sufficient to suppress tumor angiogenesis and growth in xenograft models. However, this study did not address the question whether HIFs are involved in this effect. In the present work, we were able to show that HIF is an integral factor required for efficient phosphorylation of H2AX under physiologically hypoxic conditions. Hypoxic γH2AX induction was delayed in the absence of HIF-α. We previously reported that DNA-PK expression was reduced in MEF-*Hif1a*$^{-/-}$*r*T cells (Wirthner et al., 2008), raising the possibility that DNA-PK might be the responsible kinase for H2AX phosporylation in chronic hypoxia. DNA-PK has been shown to phosphorylate H2AX in different cell lines and *in vivo* in response to DNA damage (Stiff et al., 2004; Koike et al., 2008; An et al., 2010), under hypertonic conditions (Reitsema et al., 2005) and during apoptotic DNA fragmentation (Mukherjee et al., 2006). Of note, a recent report showed DNA-PK activation in hypoxia, resulting in increased HIF dependent gene expression (Bouquet et al., 2011). These data suggest that DNA-PK might be both upstream and downstream of HIF.

In summary, our data indicate a novel DNA-DSB independent and currently unknown mechanism by which HIF downstream effectors are involved in histone H2AX phosphorylation during hypoxia. Whether increased γH2AX is preparing the cell for a DNA endangering ROS surge following reoxygenation, or whether there is a yet to be discovered novel function of γH2AX independent of DNA-DSB repair, remains to be investigated.

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## 2 Abbreviations

| | |
|---|---|
| Aβ | Amyloid beta |
| ADAM | α disintegrin and and metalloprotease domain |
| AICD | Amyloid precursor protein intracellular domain |
| APH-1 | Anterior pharynx-defective 1 |
| APLP | Amyloid precursor protein like protein |
| APP | Amyloid precursor protein |
| ARNT | Aryl hydrocarbon receptor nuclear translocator |
| BACE | γ-site APP cleaving enzyme 1 |
| bHLH | Basic helix-loop-helix |
| CAIX | Carbonic anhydrase 9 |
| CaM | Calmodulin |
| CBP | CREB-binding protein |
| ccRCC | Clear cell renal cell carcinoma |
| Cdr2 | Cerebellar degeneration-related protein 2 |
| COX-2 | Cyclooxygenase 2 |
| CTAD | C-terminal transactivation domain |
| cyps | Cyclophilins |
| DFX | Desferroxamine |
| DMOG | Dimethyloxalylglycine |
| EGL-9 | Egg-laying abnormal-9 |
| EPAS-1 | Endothelial PAS protein 1 |
| EPO | Erythropoietin |
| FAD | Familial Alzheimer disease |
| FH | Fumarate hydratase |
| FIH | Factor inhibiting HIF |
| FKBP | FK506 binding protein |
| HLF | HIF-1α like factor |
| GLUT1 | Glucose transporter 1 |
| GOE | Great oxygenation event |
| GSAP | γ-secretase acivating protein |
| HIF | Hypoxia-inducible factor |
| HRE | Hypoxia-response element |

| | |
|---|---|
| IPAS | Inhibitory PAS protein |
| MAPK | Mitogen-activated protein kinase |
| MEF | Mouse embryonic fibroblast |
| MMP9 | Matrix metalloproteinase 9 |
| mTOR | Mammalian target of rapamycin |
| NCT | Nicastrin |
| NDRG1 | N-myc downstream-regulated gene 1 |
| NFκB | Nuclear factor-kappa B |
| NICD | Notch intracellular domain |
| NO | Nitric oxide |
| NOS | Nitric oxide synthase |
| NTAD | N-terminal transactivation domain |
| ODDD | Oxygen-dependent degradation domain |
| PAS | PER-ARNT-SIM |
| PCD | Paraneoplastic cerebellar degeneration |
| Pen-2 | Presenilin enhancer 2 |
| PION | Pigeon homolog (drosophila) |
| PI3K | Phosphatidyl inositol-3 kinase |
| PKA | Protein kinase A |
| pO2 | Oxygen partial pressure |
| PPIase | Peptidyl prolyl *cis/trans* isomerase |
| pRCC | Papillary renal cell carcinoma |
| RNAi | RNA interference |
| SNP | Single nucleotide polymorphism |
| ROS | Reactive oxygen species |
| SHH | Sonic hedgehog protein |
| TAD | Transactivation domain |
| UTR | Untranslated region |

# Curriculum Vitae

**Muriel Rahel Kaufmann**

✉ Kronwiesenstrasse 68
CH-8051 Zürich

✆ +41 79 310 58 64
@ muriel.r.k@gmail.com

Born March, 30th 1984
Single, Swiss citizen

## *Experience*

| | |
|---|---|
| February 2009 – present | **University of Zürich, Institute of Physiology, Switzerland**<br>*Ph.D. candidate in the Integrative Molecular Medicine program*<br>Supervisor: Prof. R. H. Wenger, G. Camenisch "Characterization of presenilin function in the oxygen sensing pathway"<br>Expected graduation date: November 2012 |
| May 2008 – January 2009 | **Swiss Tropical Institute, Basel, Switzerland**<br>*Ph.D. candidate in molecular Epidemiology*<br>Supervisors: DVM J. Zinsstag and Prof. M. Tanner<br>"Molecular Epidemiology of Brucellosis in Kyrgyzstan" |
| January 2008 – March 2008 | **Oxford University, Medical Research Council, UK**<br>*Life Science Research Assistant and Master student in medical biology*<br>Scientific supervisor: Dr. E. M. Hammond, Oxford (ester.hammond@gmail.com)<br>"A comparison of the activation of ATM under DNA damage and hypoxia" |
| September 2006 – December 2007 | **Stanford University, Department of Radiation Oncology, CA, USA**<br>*Life Science Research Assistant and Master student in medical biology*<br>Scientific supervisor: Prof. A. J. Giaccia, Stanford<br>"The regulation of p53 in the tumor environment" |
| July 2005 – October 2005 | **Swiss Tropical Institute Basel, Switzerland**<br>*Project assistant, Department of Public Health and Epidemiology*<br>Supervisor: Prof. Mitchell G. Weiss<br>"Social, cultural and clinical dimensions of suicide and deliberate self-harm in Switzerland" |

## *Education*

| | |
|---|---|
| 2007 – 2008 | **M.Sc. in Biology, University of Zürich, Switzerland (26th of May 2008)**<br>Supervisors: Dr. Gieri Camenisch and Prof. Roland H. Wenger |
| 2005 – 2006 | **B.Sc. in Biology, University of Zürich, Switzerland (September 2006)**<br>Medical biology track |
| 2002 – 2004 | **Biology studies, University of Basel, Switzerland**<br>Physical chemistry/biophysical chemistry track |
| 1996 – 2002 | **High School, Kantonsschule Sursee, Switzerland** |

## *Technical Skills*

| | |
|---|---|
| cell culture | primary, immortalized and hybridoma cell culture, lentiviral work, bacterial work, biosafety level III |
| *in vivo* work | LTKI (accredited by FELASA) , tumor xenograft models, immunohistochemistry |
| wetlab techniques | protein analysis (immunoblotting, immunofluorescence, ELISA), DNA sequencing, cloning, RT-qPCR, luciferase assays, hypoxia work |
| *in silico* work | NCBI, UCSC genome browser, JASPAR |

## *Skills and Achievements*

| | |
|---|---|
| Languages | German (native), English (fluent, Cambridge Advanced Certificate, 2005), French (fluent), Spanish (basic), Russian (basic) |
| Teaching Skills | Supervision and teaching of undergraduate students, Lecturing in Undergraduate Summer School (BUSS 2011), Teaching of physiology practical courses |
| Memberships | Deutsche Physiologische Gesellschaft (2011 – present)<br>Swiss Physiological Society (2009 – present) |
| Computer Skills | MS Office, GraphPadPrism4, CorelDRAW X3, Clone Manager, STATA |

## *Publications*

1 Balamurugan K, Luu VD, **Kaufmann MR**, Hofmann VS, Boysen G, Barth S, Bordoli MR, Stiehl DP, Moch H, Schraml P, Wenger RH, Camenisch G, *Onconeuronal cerebellar degeneration-related antigen, Cdr2, is strongly expressed in papillary renal cell carcinoma and leads to an attenuated hypoxic response*, Oncogene, 2009, 28:3274-3285

2 Bencokova Z[#], **Kaufmann MR**[#], Pires IM, Lecane PS, Giaccia AJ, Hammond EM, *ATM activation and signaling under hypoxic conditions*, Molecular and Cellular Biology, 2009, 29:526-537.
[#] *Equal contribution*

3 Hammond EM, **Kaufmann MR**, Giaccia AJ, *Oxygen sensing and the DNA-damage response*, Current Opinion in Cell Biology, 2007, 19:680-684

## *Manuscripts Submitted / in Preparation*

1 **Kaufmann MR**, Barth S, Konietzko U, Marti HH, Camenisch G, Wenger RH, *Regulation of hypoxia-inducible factor by presenilin loss-of-function mutations*, Jounal of Neuroscience, in review 2012

2 **Kaufmann MR**, Schraml P, Moch H, Boysen G, Wenger RH, Camenisch G, *Onconeuronal cerebellar degeneration-related antigen as a diagnostic marker for papillary renal cell carcinoma*, submitted 2012

3 Wrann S[#], **Kaufmann MR**[#], Stiehl D, Wenger RH, *HIF mediated and DNA damage independent histone H2AX phosphorylation in chronic hypoxia*, submitted 2012
[#]*Equal contribution*

## *Grants and Scholarships*

| | |
|---|---|
| December 2011 | **COST action**: Scientific Mission to Cambridge University, UK (1'100 EUR) |
| November 2011 | **Keystone Symposia Scholarship** (1'200 USD) |
| September 6, 2010 | **Travel Grant** from the Swiss Physiological Society (1'400 CHF) |
| December 7, 2009 | **Travel Grant** from Hartmann-Müller-Stiftung (1'000 CHF) |
| January – March 2008 | **Scholarship** from University of Oxford as a Life Science Research Assistant |
| March – December 2007 | **Scholarship** from Stanford University as a Life Science Research Assistant |

## *Congresses and Presentations*

| | |
|---|---|
| February 12 – 17, 2012 | Keystone Symposia, Advances in hypoxic signaling, Banff, CN, **oral presentation** |
| October 9 – 14 2011 | Monte Verita conference on Hypoxia, Ticino, **poster presentation** |
| September 16 - 17, 2011 | Symposium für Junge Physiologen, Leipzig, Germany, **oral presentation** |
| August 26, 2011 | 7$^{th}$ Symposium of the ZHIP, Zürich, **oral presentation** |
| January 8 – 12, 1011 | OXYGEN2011 Conference, Davos, Switzerland, **poster presentation** |
| August 8 – 12, 2010 | ISMM Congress on High Altitude medicine, Arequipa, Peru, **poster presentation** |
| February 5 – 6, 2010 | Atmungsphysiologische Arbeitstagung, Essen, De, **oral presentation** |
| January 19 – 25, 2010 | Keystone Symposia on Hypoxia, Colorado, USA, **poster presentation** |

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